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(54) Title: TAPETUM-SPECIFIC PROMOTERS FROM *BRASSICACEAE* SPP

(57) Abstract

Tapetum-specific promoters, designated A3 and A9 and capable of driving expression of 12.9 kDa and 11.6 kDa proteins in *Arabidopsis thaliana* and related proteins within the family *Brassicaceae*, have been discovered, isolated and cloned. The promoters can be used to drive male sterility DNA such as that coding for a nuclease, protease or glucanase. Alternatively or in addition, male sterility can be achieved by disrupting the proper expression of the A3 and/or A9 genes, for example by transcribing RNA which is antisense to the RNA normally transcribed from the A3 and A9 genes, or by expressing DNA coding for a ribozyme specific for at least one of the A3 and A9 genes.

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TAPETUM-SPECIFIC PROMOTERS FROM *BRASSICACEAE* SPP.

This invention relates to the application of recombinant DNA technology to plants, specifically for the purpose of achieving male sterility.

The production of hybrids *via* sexual hybridisation of parents with differing genetic backgrounds is an important practice in modern agriculture. Due to the manifestation of heterosis or hybrid vigour the offspring are superior to the parents in such key agronomic characters as yield and disease resistance. Further, where the parents are extensively homozygous, the resulting offspring are genetically very uniform and therefore the crop behaves in an equally uniform manner in such important characteristics as germination time, height of growth, susceptibility to disease, flowering time, seed ripening time etc, which greatly improves the efficiency of crop management. For these reasons hybrid seed is attractive to the farmer and therefore sells at a premium.

In nature, the reproductive organs of many plant species are arranged in a manner that greatly favours self-fertilisation and consequently the production of non-hybrid offspring. Therefore, in order to produce hybrid seed free from contamination with selfed seed, cross-fertilisation is carried out using a variety of mechanical, chemical and genetic methods that prevent self-pollination.

An important mechanical method for hybrid seed production is available for *Zea mays*. In this species, the male and female reproductive organs are located on different parts of the same plant, which facilitates emasculation by a process known as detasseling - removal of the anthers. However, the reproductive organs of most other major crops are not so conveniently arranged making emasculation a very labour-intensive operation; as a consequence, hybrid seed produced by this method is very expensive.

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Chemical methods rely on gametocide, such as etherel, which kill or block viable pollen production. However, such chemicals are usually expensive and difficult to administer, particularly to crops with an indeterminate flowering habit.

Two commonly used genetic methods for preventing self-pollination are self-incompatibility and male sterility. In self-incompatibility systems, viable pollen is produced but a biochemical block prevents self-pollination by interfering with pollen germination or pollen tube growth. However, such systems are complicated by the scarcity of self-incompatible female lines, propagation difficulties and the frequent instability of the self-incompatibility. In some cases, the problems of propagation can be eased by chemical suppression of the self-incompatibility or by the hand pollination of immature buds ("bud pollination") before activation of the biochemical block to self-pollination. However, suppressible self-incompatibilities are often vulnerable to climatic stress which reduces the effectiveness of the system. The important crop genus *Brassica* provides a good example of the difficulties associated with self- incompatibility systems for hybrid seed production. Although self-incompatibility is widespread in *Brassica* spp., the system is complex, female lines are very difficult to propagate and the self-incompatibility is prone to breakdown under stressful climatic conditions.

In agricultural terms, the most important natural mechanism employed to prevent self-pollination is so-called male sterility. Male sterility usually results from the manifestation of certain mutations carried in the nuclear or organellar (chloroplastic and mitochondrial) genomes that result in the degeneration of the anthers or pollen prior to dehiscence (release of pollen). Plants expressing male sterility are therefore female only, and any seed produced by such plants must be the product of cross-pollination from a male fertile plant. Currently, the greatest barrier to the widespread availability of hybrid seed is the absence of effective male-sterility.

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Naturally occurring male sterility systems are available in several crops: maize, sugarbeet, oilseed rape and sunflower. Many have flaws such as the breakdown of sterility and the production of pollen under stressful climatic conditions. Genetically controlled male sterility has previously relied mostly upon the chance discovery of male sterile plants in the breeding population. The development of an effective male sterility system would remove this dependence on an unpredictable event and give more control to the plant breeder. The present invention relates to such a development.

The promoter is that region of a gene which regulates its expression, for example by specifying the time or location of expression. Promoters can be separated from the coding region of a gene and used to drive a different coding region, thus allowing the expression of a different product. A promoter can in principle be used to effect male sterility if it is specific to the cells/tissue involved in the production of male gametes. The tapetum is a specialised cell layer within the anther that plays a crucial role in the supply of nutrients to the developing microspores. Malfunction of the tapetum is the cause of many types of natural male sterility.

According to EP-A-0329308 (Paladin Hybrids Inc), many of the difficulties associated with naturally-based male-sterility and sexual incompatibility systems can be overcome by a proposed "artificial" male-sterility system. EP-A-0329308 describes several possible variants of the system. In one type, the central element is a chimeric gene consisting of a microspore- specific promoter and a male sterility DNA. Microspores are immature pollen grains. The important property of male-sterility DNA is that its expression is designed to cause the termination of microspore development, by interfering with processes unique or essential to it. Since the promoter is microspore-specific, the male-sterility DNA is transcribed into RNA only in the microspores. The application describes several types of

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male- sterility DNA, specifying: anti-sense RNA to microspore-specific genes and to proteins with a general, but essential, cell function (eg actinidin and tubulin); and the cytotoxic proteins Ricin A and diphtheria toxin.

However, the system as described appears to have a serious drawback, in that the teaching of EP-A-0329308 would not produce plants which are necessarily male-sterile plants. Since the action of the microspore-specific promoter described in the invention occurs post-meiosis, segregation of the male sterility DNA in the microsporocytes of plants heterozygous for the factor would result in only half the pollen grains receiving the factor.

A possible way to circumvent the segregation problem would be to generate plants homozygous for the sterility factor. However, propagation of such a plant would have to proceed via asexual processes since any pollination would again return the offspring to heterozygosity. This limits the application of the invention to plant species where such propagation is commercially viable. It would therefore be desirable for male sterility DNA to be expressed in the mother tissue thereby to affect all the pollen grains.

EP-A-0344029 (Plant Genetic Systems (PGS)) also describes an artificial male sterility system for use in hybrid seed production. It is based on a chimeric gene consisting of an anther-specific promoter (from the gene designated TA29) isolated from *Nicotiana tabacum* and a male sterility DNA. In this case, the promoters are derived not from microspore-specific genes as in the Paladin system, but from genes expressed exclusively within the tapetum. Hence, the chimeric gene is designed to prevent microspore development and cause male-sterility by disrupting or destroying the tapetum.

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The most important difference between this approach and that of EP-A-0329308, is that the choice of a promoter which is active in a cell-type not subject to meiosis, avoids the problems associated with genetic segregation. Destruction of the tapetum prevents the maturation of all microspores regardless of genetic make-up.

As part of the continuing endeavour to improve the means available to the plant geneticist for inducing male sterility in commercially important crops and other plants, the identification of further useful genes and associated promoters is actively sought. Among the commercially most significant crops in the world today are included members of the family *Brassicaceae*, particularly *Brassica napus*, commonly known as oil-seed rape. If tapetum-specific genes and promoters from members of the family *Brassicaceae* could be elucidated, plant breeders would have at their disposal powerful tools to use in the development of male sterile *B. napus* and other members of the *Brassicaceae* family. There is an attraction in being able to keep heterologous DNA within the family or smaller taxonomic division, as unpredicted effects may be reduced or minimised. Further, transgenic members of the family *Brassicaceae* incorporating heterologous DNA from other members of the same family may well be more acceptable from the regulatory point of view than *Brassicaceae* family members incorporating DNA from more remote sources.

The present invention is based on the discovery of novel and useful genes and promoters from the family *Brassicaceae* and relates to methods, genetic constructs and transgenic plants harnessing the discovery.

According to a first aspect of the present invention, there is provided a recombinant or isolated DNA molecule comprising a promoter which naturally drives expression of a gene encoding an 11.6 or 12.9 kDa tapetum protein in

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Arabidopsis thaliana or an equivalent protein in another member of the family *Brassicaceae*.

In this specification, the gene encoding the 12.9 kDa protein in *A. thaliana* and equivalents of that gene in other members of the family *Brassicaceae* will be referred to as the A3 gene; the gene encoding the 11.6 kDa protein in *A. thaliana* and equivalents of that gene in other members of the family *Brassicaceae* will be referred to as the A9 gene. The A9 and A3 are different in sequence and pattern of expression from the tapetum-specific promoters described in EP-A-0344029. Induction by external factors such as heat shock or herbicide application, as is described in one embodiment of EP-A-0329308, is not required for this invention to function, so it need have none of the attendant problems with, for example, indeterminate flowering habits.

A3 temporal expression spans the period in anther development when the microsporocytes are in meiotic division to early microspore interphase. A9 gene is expressed in tapetal cells. Expression of A9 is initiated in anthers that contain meiocytes and continues into anthers that contain microspores in early first interphase.

The molecular weights quoted above are putative and derived from the number of amino acids believed to be present, as deduced from the DNA sequence. The 12.9 kDa protein encoded by the A3 gene of *A. thaliana* has 118 amino acids; the 11.6 kDa protein encoded by the A9 gene of *A. thaliana* has 107 amino acids. It will therefore be appreciated that the molecular weights refer to the un-glycosylated protein. In addition, the effect on any other post-translational processing such as partial proteolysis is discounted.

Although figures given above relate only to proteins of *A. thaliana*, those skilled in the art will readily be able to identify equivalent proteins from other members

of the family *Brassicaceae*. For example, the equivalent A9 gene in *Brassica napus* encodes a putative protein of 96 amino acids in length having a calculated molecular weight of 10.3 kDa. Such equivalent genes may be identified by hybridisation studies, restriction fragment length polymorphism (RFLP) and other methods known in the art. Genes encoding closely equivalent proteins may for example hybridise under stringent conditions (such as at approximately 35°C to 65°C in a salt solution of approximately 0.9 molar) to the *A. thaliana* A3 and A9 genes, or fragments of them of, for example, 10, 20, 50 or 100 nucleotides. A 15-20 nucleotide probe would be appropriate under many circumstances.

The preferred A3 and A9 promoters described in this specification are from *Arabidopsis thaliana* and can be isolated by methods known in the art, for example by (a) synthesising cDNA from mRNA isolated from the stamens of the plant *Brassica napus*, (b) isolating this cDNA, (c) using this cDNA as a probe to identify regions of the plant genome of *Arabidopsis thaliana* that encode stamen-specific mRNA and (d) identifying the upstream (5') regulatory regions that contain the promoter of this DNA. This procedure also demonstrates that probes based on, or derived from, the coding regions of a stamen-specific DNA from one species of plant may be used to isolate DNA sequences encoding stamen-specific mRNAs from other species. A3 and A9 promoters from other members of the family *Brassicaceae*, for example from *B. napus* itself, are also included within the scope of the invention, as are those which include non-essential variations from the natural sequences.

Particularly preferred promoters are those upstream of the coding regions of the sequences shown in Figure 4 (for the *A. thaliana* A3 gene) and Figure 7 (for the *A. thaliana* A9 gene) as will subsequently be described in the examples. Those skilled in the art will be able to identify with sufficient precision the promoters driving the coding regions and to isolate and/or recombine DNA containing them.

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Promoter-containing DNA in accordance with the invention can be used to confer male sterility on plants, particularly those belonging to the family *Brassicaceae*, in a variety of ways as will be discussed below. In an important embodiment of the invention, therefore, a promoter as described above is operatively linked to DNA which, when expressed, causes male sterility.

Since an effective sterility system is complete, propagation of the seed parent must proceed either by asexual means or via the pollination of the male-sterile by an isogenic male-fertile line, and the subsequent identification or selection of male sterile plants among the offspring. Where vegetative propagation is practical, the present invention forms a complete system for hybrid production. Where fertility restoration is necessary to produce a seed crop, the present invention forms the basis of a new male sterility system. In some seed crops where the level of cross pollination is high, seed mixtures may enable restoration to be bypassed. The male sterility will be particularly useful in crops where restoration of fertility is not required, such as in the vegetable *Brassica* spp., and such other edible plants as lettuce, spinach, and onions.

DNA in accordance with the invention and incorporating the A3 and/or A9 promoter can drive male sterility DNA thereby producing male sterile plants, which can be used in hybrid production. The promoters are highly tapetum specific and so the sterility DNA is only expressed in the tapetum. The control of expression is very strong and the DNA is not expressed in other cells of the plant. The system prevents the production of viable pollen grains. All transformed plants and their progeny are male sterile; there is no problem with meiotic segregation.

A construct comprising a promoter operatively linked to a male sterility DNA can be transformed into plants (particularly those of the genus *Brassica*, but also other genera such as *Nicotiana* and *Hordeum*) by methods which may be well known in

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themselves. This transformation results in the production of plants, the cells of which contain a foreign chimeric DNA sequence composed of the promoter and a male sterility DNA. Male-sterility DNA encodes an RNA, protein or polypeptide which, when produced or over-produced in a stamen cell of the plant, prevents the normal development of the stamen cell.

The tapetum specific promoters may be used to drive a variety of male sterility DNA sequences which code for RNAs proteins or polypeptides which bring about the failure of mechanisms to produce viable male gametes. The invention is not limited by the sequence driven, but a number of classes and particular examples of male sterility promoter-drivable sequences are preferred.

For example, the drivable male sterility DNA may encode a lytic enzyme. The lytic enzyme may cause lysis of one or more biologically important molecules, such as macromolecules including nucleic acid, protein (or glycoprotein), carbohydrate and in some circumstances lipid.

Ribonuclease (such as RNase T1) and barnase are examples of enzymes which cause lysis of RNA. Examples of enzymes which lyse DNA include exonucleases and endonucleases, whether site-specific such as *Eco*RI or non-site-specific. Glucanase is an example of an enzyme which causes lysis of a carbohydrate. The enzyme glucanase is naturally produced in anthers where it functions to release the young microspores from a protective coat of poly-glucan laid down before meiosis. The appearance of the enzyme activity is developmentally regulated to coincide with the correct stage of microspore development. One important attraction of glucanase as a potential sterility DNA is that plants are found in nature that are male-sterile due to mutations causing mistiming of glucanase expression and the destruction of the microspores. Two types are recognised depending on whether the appearance of glucanase activity is premature or late. The expression of many

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genes, including those expressed within the anther, exhibit various patterns of temporal regulation. Therefore, in order to use glucanase as a sterility DNA, the promoter chosen to drive expression of the gene must provide an appropriate developmental regulation of glucanase activity, preferably by mimicking the pattern of expression found in association with natural male-sterility. One means of achieving male sterility is to isolate the promoter from a tapetum-specific gene with the same pattern of expression as found for glucanase activity in male-sterile mutant plants. Since late expression of a glucanase is unlikely to produce sterility in plants with a functional anther glucanase gene, the sterility factor would require a promoter capable of driving transcription before the appearance of normal glucanase activity. In the RM *cms* mutant of *Petunia* (Izhar, S. and Frankel, R. *Theor. Appl. Genet.*, 41 104-108 (1971)) glucanase expression within the anther first appears at the end of meiotic prophase, and increases to a maximum by the completion of meiosis. This pattern of expression contrasts with that in normal *Petunia* plants, where glucanase activity within the anthers appears concomitantly with the breakdown of the tetrads and the release of the young microspores. The aberrant pattern of glucanase activity found in the *cms* mutant is thought to be responsible for the destruction of the microspores and male sterility. Thus, to mimic this mutation using a sterility DNA encoding a glucanase enzyme requires a promoter capable of driving transcription of the male sterility DNA within the anthers, and preferably within the tapetum, during the phase of anther development between prophase of meiosis and the appearance of the tetrad of microspores; the A3 and A9 promoters discussed above are therefore well suited to drive this gene. A tapetum-specific (or at least anther-specific) promoter is also advantageous since β(1,3)-glucans are found elsewhere within plants, for example in phloem sieve elements, where they presumably perform essential functions.

The spatial regulation of the enzyme should also ensure access to the target cells. Secretion into the locular space is ensured by the provision in a preferred

embodiment, of a suitable signal sequence in a translational fusion with the glucanase coding sequence.

DNA encoding glucanase is advantageous as male sterility DNA, as it has no product which is cytotoxic outside the target cell. Glucanase as a male sterility DNA mimics natural systems and is inherently less destructive than for example ribonuclease, and so does not present such a problem if the promoter is slightly active in certain conditions in other cell types.

Actinidin is an example of a protease, DNA coding for which can be suitable male sterility DNA. Other examples include papain zymogen and papain active protein.

Lipases whose corresponding nucleic acids may be useful as male sterility DNAs include phospholipase A_2.

Male sterility DNA does not have to encode a lytic enzyme. Other examples of male sterility DNA encode enzymes which catalyse the synthesis of phytohormones, such as isopentyl transferase, which is involved in cytokinin synthesis, and one or more of the enzymes involved in the synthesis of auxin. DNA coding for a lipoxygenase or other enzymes having a deleterious effect may also be used.

Other male sterility DNAs include antisense sequences. Introducing the coding region of a gene in the reverse orientation to that found in nature can result in the down-regulation of the gene and hence the production of less or none of the gene product. The RNA transcribed from antisense DNA is capable of binding to, and destroying the function of, a sense RNA version of the sequence normally found in the cell thereby disrupting function. Examples of such anti-sense DNAs are the anti-sense DNAs of the A3 and A9 genes which may be produced in the anther

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under control of the A3 and A9 promoter. Since these genes are normally expressed in the tapetum, antisense to them may be expected to disrupt tapetal function and result in male sterility.

It is not crucial for antisense DNA solely to be transcribed at the time when the natural sense transcription product is being produced. Antisense RNA will in general only bind when its sense complementary strand, and so will only have its toxic effect when the sense RNA is transcribed. Antisense DNA corresponding to some or all of the DNA encoding the A3 or A9 gene products may therefore be produced not only while the A3 and A9 genes are being expressed. Such antisense DNA may be expressed constitutively, under the control of any appropriate promoter.

According to a further aspect of the invention, therefore, there is provided antisense nucleic acid which includes a transcribable strand of DNA complementary to at least part of the strand of DNA that is naturally transcribed in a gene encoding an 11.6 or 12.9 kDa tapetum protein in *Arabidopsis thaliana* or an equivalent protein in another member of the family *Brassicaceae*.

Antisense DNA in accordance with this aspect of the invention may be under the control of any suitable promoter which permits transcription during, but not necessarily only during, tapetum development. As indicated above, the promoter may therefore be constitutive, but the use of tapetum-specific promoters such as A3 and A9 as described above in relation to the first aspect of the invention is certainly not excluded and may be preferred for even greater control. Such antisense DNA would generally be useful in conferring male sterility on members of the family *Brassicaceae*.

A still further example of male sterility DNA encodes an RNA enzyme (known as a ribozyme) capable of highly specific cleavage against a given target sequence (Haseloff and Gerlach *Nature* 334 585-591 (1988). Like antisense DNA, ribozyme DNA (coding in this instance for a ribozyme which is targeted against the RNA encoded by the A3 or A9 gene) does not have to be expressed only at the time of expression of the A3 and A9 genes. Again, it may be possible to use any appropriate promoter to drive ribozyme-encoding DNA, including one which is adapted for constitutive expression.

According to a further aspect of the invention, there is therefore provided DNA encoding a ribozyme capable of specific cleavage of RNA encoded by a gene encoding an 11.6 or 12.9 kDa tapetum protein in *Arabidopsis thaliana* or an equivalent protein in another member of the family *Brassicaceae*. Such ribozyme-encoding DNA would generally be useful in conferring male sterility on members of the family *Brassicaceae*.

In preferred embodiments of DNA sequences of this invention, including those comprising the A3/A9 promoter-male sterility DNA construct, 3' transcription regulation signals, including a polyadenylation signal, may be provided. Preferred 3' transcription regulation signals are derived from the Cauliflower Mosaic Virus 35S gene. It should be recognised that other 3' transcription regulation signals could also be used.

The antisense nucleic acid and ribozyme-encoding nucleic acid described above are examples of a more general principle: according to another aspect of the invention, there is provided DNA which causes (for example on its expression) selective disruption of the proper expression of the A3 and A9 genes.

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Recombinant DNA in accordance with the invention may be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected (or transformed: the terms are used interchangeably in this specification) with them and, preferably, to enable selection of cells harbouring vectors incorporating heterologous DNA. Appropriate start and stop signals will generally be present. Additionally, if the vector is intended for expression, sufficient regulatory sequences to drive expression will be present; however, DNA in accordance with the invention will generally be expressed in plant cells, and so microbial host expression would not be among the primary objectives of the invention, although it is not ruled out. Vectors not including regulatory sequences are useful as cloning vectors.

Cloning vectors can be introduced into *E. coli* or another suitable host which facilitate their manipulation. According to another aspect of the invention, there is therefore provided a host cell transfected or transformed with DNA as described above.

DNA in accordance with the invention can be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, but recombinant DNA technology forms the method of choice.

Ultimately, DNA in accordance with the invention (whether (i) A3/A9 promoter plus male sterility gene, (ii) antisense DNA to A3/A9 gene or ribozyme DNA targeted to A3/A9 RNA) will be introduced into plant cells, by any suitable means. According to a further aspect of the invention, there is provided a plant cell including DNA in accordance with the invention as described above.

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Preferably, DNA is transformed into plant cells using a disarmed Ti-plasmid vector and carried by *Agrobacterium* by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. Alternatively, the foreign DNA could be introduced directly into plant cells using an electrical discharge apparatus. This method is preferred where *Agrobacterium* is ineffective, for example where the recipient plant is monocotyledonous. Any other method that provides for the stable incorporation of the DNA within the nuclear DNA of any plant cell of any species would also be suitable. This includes species of plant which are not currently capable of genetic transformation.

Preferably DNA in accordance with the invention also contains a second chimeric gene (a "marker" gene) that enables a transformed plant containing the foreign DNA to be easily distinguished from other plants that do not contain the foreign DNA. Examples of such a marker gene include antibiotic resistance (Herrera-Estrella *et al*, 1983), herbicide resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029). Expression of the marker gene is preferably controlled by a second promoter which allows expression in cells other than the tapetum, thus allowing selection of cells or tissue containing the marker at any stage of regeneration of the plant. The preferred second promoter is derived from the gene which encodes the 35S subunit of Cauliflower Mosaic Virus (CaMV) coat protein. However any other suitable second promoter could be used.

A whole plant can be regenerated from a single transformed plant cell, and the invention therefore provides transgenic plants (or parts of them, such as propagating material) including DNA in accordance with the invention as described above. The regeneration can proceed by known methods. When the transformed plant flowers it can be seen to be male sterile by the inability to produce viable pollen. Where pollen is produced it can be confirmed to be non-viable by the inability to effect seed set on a recipient plant.

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The invention will now be illustrated by the following Examples. The following restriction enzyme and other abbreviations are used:

A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hinc*II; Hd, *Hind*III; K, *Kpn*I; M, *Mlu*I; N, *Not*I; Nc, *Nco*I; Nr, *Nru*I; P, *Pst*I; R, *Rsa*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sst*I; Sa, *Sal*I; Sp, *Sph*I; Sm, *Sma*I; Ss, *Ssp*I; SII, *Sac*II; X, *Xho*I; Xb, *Xba*I.

ORF = open reading frame

The Examples refer to the accompanying drawings, in which:

FIGURE 1 shows the DNA sequence of the *B. napus* cDNA A3 together with the deduced protein sequence of the ORF contained in A3;

FIGURE 2a shows a comparison of the DNA sequences of the *B. napus* cDNAs E3 and E3 with the *A. thaliana* A3 gene. The underlined trinucleotides indicate the end of the ORF encoded by each sequence;

FIGURE 2b shows a comparison of the putative polypeptides encoded by *B. napus* cDNAs E3 and E5 with that encoded by the *A. thaliana* A3 gene;

FIGURE 3 shows a restriction enzyme map of the *A. thaliana* genomic clone G3.6. Only relevant sites are shown and these may not be unique in G3.6. The position of the coding region of A3 is indicated as a filled box. Also the extent of inserts cloned into the plasmids pRS5 and 15 is shown;

FIGURE 4 shows the DNA sequence and putative primary structure of the *A. thaliana* A3 gene. The underlined sequence is conforms to a TATA box motif;

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FIGURE 5 shows the DNA sequence of the *B. napus* cDNA A9 and the putative primary structure of the ORF contained in the cDNA;

FIGURE 6 shows a restriction map of the *A. thaliana* genomic clone G9.1. The position of the A9 coding region is shown as a filled box and the extent of inserts in the plasmids pWP39, 55 and 64 is indicated;

FIGURE 7 shows the DNA sequence and putative primary structure of the *A. thaliana* A9 gene. The underlined sequence is conforms to a TATA box motif;

FIGURE 8a shows the DNA sequence homology between the *B. napus* A9 cDNA and the *A. thaliana* A9 gene. Underlined nucleotides indicate the position of stop codons for the ORFs contained in these sequences;

FIGURE 8b shows the homology between the putative products encoded by the *B. napus* A9 cDNA and the *A. thaliana* A9 gene;

FIGURE 9 shows the construction of a chimeric gene containing a transcriptional fusion between the A3 promoter and an *E. coli* gene encoding β-glucuronidase;

FIGURE 10 shows the construction of chimeric genes containing transcriptional fusions between the A9 promoter and an *E. coli* gene encoding β-glucuronidase;

FIGURE 11 shows β-glucuronidase activity in anthers of A9-GUS transformed tobacco plants;

FIGURE 12 shows the construction of intermediate cloning vectors used in the production of chimeric genes that express sense and anti-sense RNA from the A3 and A9 promoters in transgenic plants.

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FIGURES 13a and 13b show the construction of chimeric genes between the A3 and A9 promoters and the RNAse barnase; and

FIGURES 14a and 14b show the construction of chimeric genes between the A3 and A9 promoters and a *N. tabacum* β-1,3 glucanase gene which lacks a C-terminal extension. Figure 14a illustrates the preparation of transcriptional fusion constructs and Figure 14b illustrates the preparation of translational fusion constructs.

In the Examples, unless stated otherwise, all procedures for making and manipulating recombinant DNA were carried out using standard procedures described in Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982.

EXAMPLES

Example 1 Isolation of the anther-specific genes A9 and A3 from *Arabidopsis thaliana*

Anther-specific cDNAs were isolated by differential screening of *Brassica napus* cDNA libraries constructed from RNA extracted from dissected anthers, as described below. cDNA clones A3 and A9 were isolated from a library constructed from anthers that were 1.4-1.8 mm in length. This library was constructed in the vector Lambda Zap (Stratagene). The A3 and A9 cDNAs were used as probes to isolate homologous genes from an *A.thaliana* genomic library constructed in the vector Lambda Dash (Stratagene).

Materials and methods

Plant material. All seeding material for nucleic acid isolation was obtained from 2-3 week old plants grown in a controlled environment growth cabinet with 18h photoperiod at 24°C. Seedling RNA for differential screening and Northern blot analysis was obtained from *B. napus oleifera* var "Topaz". Male fertile buds were collected from field grown plants of *B. napus oleifera* var. "Lictor" (Nickersons Seeds, Cambridge, UK). Male-sterile buds were obtained from field grown *B. napus* var. CMS "Ogura" (Nickersons Seeds, Cambridge, UK) plants.

Dissection of anthers. For cDNA library construction, flower spikes were quickly harvested and kept at 4°C until required, but no longer than 5h. Anthers were dissected from appropriately sized buds using fine forceps and immediately frozen in liquid nitrogen.

Pollen isolation. Microspores were isolated from fresh buds of the appropriate length using the method of Choung and Beaversdorf (*Plant Sci.* **39** 219-226 (1985)), and frozen at -80°C prior to RNA isolation. (Pollen isolated from frozen buds yielded only highly degraded RNA).

Collection of buds. Large samples of complete whorls of buds, at a stage immediately prior to the opening of first flowers, were frozen in liquid nitrogen and stored at -80°C.

Cytological staging of anthers and buds. The developmental stage of buds of predetermined length was assessed by light microscopic examination of sporogenous cells, microspores or pollen grains extruded from whole anthers squashed in the presence of aceto-orcein or acridine orange. Accurate determination of bud length was performed using a low-powered light microscope

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equipped with a calibrated eyepiece graticule. Bud lengths stated were measured from the base of the pedicle to the tip of the outermost sepal.

RNA isolation and analysis. Material intended for low resolution Northern dot blot analysis or for mRNA isolation was ground to a fine powder in a mortar cooled with liquid nitrogen. Total RNA was isolated from the powder using a phenol based method as described previously (Draper *et al.*, "Plant Genetic Transformation and Gene Expression: A Laboratory Manual", Blackwell Scientific Publishers, Oxford (1988)). Poly(A)$^+$ RNA was purified by two rounds of oligo(dT)-cellulose chromatography essentially as described in the Maniatis et al manual. RNA for high resolution dot blots was isolated according to the method of Verwoerd *et al.*, *Nuc. Acids Res.* 17 2362 (1989)).

cDNA library construction and screening. cDNAs were synthesised from poly(A)$^+$ RNA using (Amersham or Pharmacia) cDNA synthesis kits, according to the manufacturers instructions. cDNAs were ligated into *Eco*RI cleaved dephosphorylated lambda Zap I (Stratagene) ("sporogenesis" library) or lambda Zap II (Stratagene) ("microspore-development" library) and packaged using Amersham *in vitro* packaging extracts. Clones were screened differentially, on duplicate HYBOND-N filters (Amersham) with [^{32}P]-labelled single-stranded cDNA probe prepared from either the appropriate anther poly(a)$^+$ RNA or seedling poly(A)$^+$ RNA according to Sargent *Methods in Enzymol.* 152 423-432 (1987)). (The expression HYBOND-N is a trade mark.)

RNA dot and gel blots. Total RNA for dot-blots was spotted onto HYBOND N (Amersham) according to the manufacturers instructions. Northern gels were run and RNA transferred to HYBOND-N according to Fourney (*BRL Focus* 10 5-7 (1988)). Hybridisation and washing of HYBOND-N filters was according to manufacturers instructions.

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In situ hybridisation. For embedding and sectioning *B. napus* buds were frozen in CRYO-M-BED (TAAB Laboratories Equipment Ltd). (The expression CRYO-M-BED is a trade mark.) Sections were cut nominally 10 μm thick, mounted on subbed slides (Van Prooijen-Knegt *et al.*, *Histochemical J.* 14 333-344 (1983)) fixed in 4% paraformaldehyde and dehydrated. [^{35}S]rUTP (>1000 Ci/mmol, Amersham SJ.1303) labelled sense and anti-sense RNA probes were transcribed from the T3 and T7 promoters of BLUESCRIPT SK$^-$ (Stratagene), in which the cDNAs are cloned. (The expression BLUESCRIPT SK$^-$ is a trade mark.) Following transcription, probes were cleaved by alkaline hydrolysis to generate probe fragments approximately 150bp in length. The hybridisation solution was 50% formamide, 300mM NaCl, 10mM Na_2HPO_4 pH 6.8, 10mM Tris-HCl pH 7.5, 5mM EDTA, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10mM dithiothreitol, 10% dextran sulphate, 0.7mg/ml *E.coli* tRNA, 50-100ng/ml probe stock ($6.7x10^5$ cpm/ng probe). Sections were hybridised in 30 μl hybridisation solution at 50°C for 16h. Slides were washed 3x1h at 50°C in 50% formamide, 300mM NaCl, 10mM Na_2HPO_4 pH 6.8, 10mM Tris-HCl pH 7.5 and then rinsed in RNase A buffer to remove formamide. RNase A treatment, (150 μg/ml RNase A in 500mM NaCl, 10mM Tris HCl pH 7.5), was carried out at 37°C for 1h. The slides were then washed twice in 2xSSC (0.3M NaCl, 0.03M Na citrate, pH 7.0) at 65°C for 30 min, dehydrated through graded alcohols and dried. For autoradiography, slides were dipped at 45°C in ILFORD K5 nuclear track emulsion (1g/ml in 1:59 glycerol:water mix). (The expression ILFORD K5 is a trade mark.) Exposure time was between 2 and 14 days. Development was in KODAK D19. (The expression KODAK D19 is a trade mark.) Following development sections were stained with methylene blue and made permanent.

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a) Isolation and characterisation of the *A. thaliana* A3 gene

Northern hybridisation analysis using RNA extracted from *B. napus* anthers, pollen, carpels and seedlings indicated that A3 was only expressed in anthers of length 1.6-2.3mm with maximal expression between 1.8-2.3mm. Thus A3 temporal expression spans the period in anther development when the microsporocytes are in meiotic division to early microspore interphase. *In situ* hybridisations suggest that in *B. napus*, A3 is expressed solely in the tapetum of the anther. The A3 cDNA is 347 bp in length and contains an open-reading frame (ORF) extending from position 1-329 bp (Fig 1) suggesting that this clone is not full-length. The estimated size of *B. napus* A3 mRNA from Northern gel blots is about 500 bp, and again suggesting that this clone is not full length. The A3 cDNA was used to isolate homologous cDNA clones (E3 and E5) from the same *B. napus* library. E5 cDNA is 422 bp long and contains an ORF from position 1-333 (Fig.2a). This cDNA is identical to A3 cDNA over the region they overlap and extends the A3 sequence 5 bp 5' of the start of A3 and 69 bp 3' of the end of A3. The E3 cDNA is 398 bp in length with an ORF extending from position 1-314 bp (Fig.2a). The E3 cDNA is 95% identical to the E5 cDNA at the nucleotide level and the putative ORF products are 91% identical. The cloning of E5 which is homologous but not identical to A3 is evidence that probes based on the sequence of the anther-specific gene A3 would enable the cloning of homologous anther-specific genes.

A 15 kb *A. thaliana* genomic clone (G3.6) was isolated that hybridises to A3 cDNA (Fig.3). The 2300 bp *Hin*dIII-*Hin*cII region which hybridised strongly with the A3 probe, was subcloned from G3.6 and partially sequenced (Fig.4) revealing an ORF (positions 770-1126) that is highly homologous to the *B. napus* E5 cDNA (82% at the nucleotide level and 76% at the protein level) (Fig.2a,b). The *A. thaliana* A3 gene does not apparently contain introns. The putative protein encoded

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by A3 consists of 118 amino-acids with a molecular mass of 12.9 kDa. A search of the NBRF protein database (release 34) did not reveal any proteins homologous to the putative A3 protein. There is a TATA box consensus sequence (Joshi, 1987) between positions 699-707 bp, 63 bp upstream of the putative start of the A3 coding region.

b) Isolation and characterisation of the A9 gene

Northern analysis and *in situ* hybridisations indicate that the *B. napus* A9 gene is expressed in the tapetal cells of anthers of length 1.5-2.3mm, with maximal expression between 2.0-2.3mm. Expression of A9 is initiated in anthers that contain meiocytes and continues into anthers that contain microspores in early first interphase. The A9 cDNA is 490 bp in length containing an ORF from position 1-296 bp (Fig.5). From Northern gel blots, the estimated size of the A9 mRNA in *B. napus* is about 550-600 bp. The abundance of the A9 mRNA was estimated at between 0.1 - 0.2 % of total anther polyA+ mRNA.

A 13 Kb *A. thaliana* genomic clone (G9.1) was isolated that hybridised to the A9 cDNA (Fig.6) and a 3145 bp *Xba*I fragment cloned and partially sequenced (Fig.7). This fragment contains an ORF at position 1461-1781 that is 76% identical to the A9 cDNA ORF at the nucleotide level (Fig.8a) and the putative products of these ORFs are 73% identical (Fig.8b). Comparison of the cDNA and genomic sequences suggests that the ORF in the cDNA starts at position 9 bp (Fig.5) and that the A9 gene contains no introns. 70 bp upstream of the putative start of the A9 gene (positions 1382-1389 bp) is a TATA box conforming to the consensus sequence of Joshi (1987). The putative ORF encoded by the *B. napus* cDNA is 96 amino-acids in length with a calculated molecular mass of 10.3 kDa and that of the *A. thaliana* gene 107 amino-acids with a mass of 11.6 kDa. Although no overall homology was found to the putative A9 proteins by searching

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the NBRF protein database the A9 protein contains a cysteine motif that is present in several 2S plant storage proteins and in some plant protease inhibitors.

Example 2 The use of the A9 and A3 promoters to drive the expression of β-glucuronidase in anthers of *Arabidopsis thaliana* and *Nicotiana tabacum*.

To demonstrate that the putative promoter regions of A3 and A9 are capable of driving the expression of a foreign gene in *A. thaliana* and *N. tabacum*, transcriptional fusions of the promoters were made to the *Escherichia coli* gene encoding β-glucuronidase (GUS).

a) A3 -GUS fusion (Fig.9)

The 1030 bp *Hinc*II fragment of G3.6 is subcloned between into the *Hinc*II site of the vector pTZ18 (Pharmacia Ltd.) forming pRS5 (Fig.9). This is cut with *Sst*I and the fragment cloned into *Sst*I-cut pIC19H (Marsh *et al.*, *Gene* 32 481-485 (1984)) forming pWP87. The A3 promoter is then recovered as a *Hin*dIII-*Nru*I fragment from pWP87 and cloned into *Hin*dIII, *Sma*I-cut pBI101.1 (Jefferson *et al.*, *EMBO J* 6 3901 (1987)). The resultant plasmid (pWP92) (Fig.9) contains 745 bp of A3 sequence upstream of the SstI site (position 745 bp in Fig.4) fused to GUS.

b) A9-GUS fusions (Fig.10)

The 329 bp *Hinc*II-*Rsa*I fragment (positions 1105-1434 bp in Fig.7) was cloned into *Hinc*II-cut pTZ18 forming pWP70A. DNA sequence analysis revealed the loss of a 'G' residue at the *Rsa*I, *Hinc*II junction which resulted in the recreation of the *Rsa*I site. The *Hin*dIII, *Bam*HI fragment of pWP70A containing the A9 promoter was cloned into *Bam*HI, *Hin*dIII-cut pBluescript (Stratagene) forming

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pWP71. To reconstruct plasmids with larger A9 upstream regions the *Eco*RI, *Hin*dIII fragment of pWP71 was replaced with the 900 bp *Hin*dIII, *Eco*RI fragment of pWP64 (Fig.6) (which contains a 1486 bp *Acc*I, *Bgl*II fragment cloned into *Acc*I, *Bam*HI-cut pTZ19) forming pWP72. Also the *Eco*RI, *Hin*dIII fragment of pWP71 was replaced with the 1397 bp *Hin*dIII, *Eco*RI fragment of pWP55 (Fig.6) (which contains a 3146 bp *Xba*I fragment cloned into *Xba*I-cut pTZ19) forming pWP73. The *Hin*dIII, *Xba*I fragments of pWP71, pWP72 and pWP73 were cloned into *Hin*dIII, *Xba*I-cut pBI101.1 forming pWP74, pWP75 and pWP76 respectively. Thus pWP74 contains a 329 bp A9 promoter fragment (positions 1108 - 1437 bp), pWP75 a 936 bp A9 fragment (positions 501-1437 bp) and pWP76 a 1437 bp A9 fragment (positions 1-1437 bp) all fused to GUS.

All the GUS constructs are then transformed into *N. tabacum* and *A. thaliana* using standard transformation techniques. Analysis of transformed plants demonstrated that GUS activity was localised to anther tissues, specifically to tapetal cells. The temporal regulation of GUS activity was identical to the temporal expression observed for the A3 and A9 genes as described in Example 1. Figure 11 shows the activity of the A9-GUS fusions in the anthers of transgenic tobacco plants. GUS activity was assayed fluorometrically in the anthers staged precisely in terms of the development of the sporogenous cells. The pattern of expression of GUS was the same (quantitatively and qualitatively) irrespective of the length of upstream region employed in the fusion. These experiments clearly demonstrate that the A9 promoter drives transcription in tapetal cells through a period commencing at the meiocyte stage of development and terminating during early microspore interphase.

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Example 3 The construction of expression cassettes and their use in producing sense and anti-sense RNA to anther-specific messages in transgenic plants.

Either anther-specific or constitutive promoters can be used to drive the expression of sense or anti-sense RNA corresponding to anther-specific transcripts in transgenic plants, thus potentially creating anther mutations and male-sterility. The same anther-specific promoters can be used to drive the anther-specific expression of genes encoding proteins or enzymes detrimental to anther function thereby creating male-sterility. The use of the expression cassettes, the construction of which is described in this example, for this application are described in Examples 4 and 5.

a) Construction of an intermediate vector to express sense and anti-sense RNA utilising the A9 promoter.

pWP72 (Fig.10) is digested with *Xba*I and religated, thus removing the *Bam*HI site in the polylinker, forming pWP78 (Fig.12). The *Kpn*I, *Sst*I (the *Sst*I end rendered blunt with Klenow) A9 promoter fragment of pWP78 is ligated into *Kpn*I, *Sma*I-cut pJIT60 forming pWP80 (Fig.12). pJIT60 is identical to pJIT30 (Guerineau *et al.*, *Plant Mol. Biol.* 15, 127-136 (1990)) except that the CaMV 35S promoter is replaced by a double CaMV 35S promoter (Guerineau *et al.*, *Nuc. Acids Res.* 16 (23) 11380 (1988)). The pWP80 intermediate vector consists of a 936 bp A9 promoter fragment fused to a polylinker derived from pBluescript with a 35S CaMV polyadenylation signal to stabilise the transcript.

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b) Construction of an intermediate vector to express sense and anti-sense RNA utilising the A3 promoter.

The CaMV promoter of pJIT60 is replaced with the A3 promoter by cloning the 745 bp *Kpn*I, *Hin*dIII fragment of pWP87 (Fig.9) into *Kpn*I, *Hin*dIII-cut pJIT60 forming pWP88 (Fig.12). pWP80 and pWP88 are therefore identical apart from the promoter region and surrounding restriction enzyme sites.

c) Construction of chimeric genes containing the tapetum-specific A9 promoter linked to the sense or anti-sense orientation of the A9 cDNA.

Anther-specific *B. napus* cDNAs were cloned into *Eco*RI-cut Lambda ZapII by the addition of *Eco*RI linkers (Pharmacia Ltd) to the ends of the cDNA. These linkers also contain internal *Not*I sites, so the entire cDNA can be recovered as a *Not*I fragment provided the cDNA contains no internal *Not*I sites. The *B. napus* cDNA for A9 is therefore recovered as a *Not*I fragment and cloned in both orientations (sense and anti-sense) into *Not*I-cut pWP80. The promoter, cDNA and terminator regions are excised from the pWP80 derivatives with a *Hin*dIII, *Xho*I digest and are cloned into *Sal*I, *Hin*dIII-cut pBin19 (Bevan *et al.*, *Nuc. Acids Res.* 22 8711-8721 (1984)).

The pBin19 derivatives are transformed into *B. napus*. The resulting transgenic plants expressing anti-sense A9 RNA are male sterile.

Other chimeric genes that can be constructed to produce male sterility are:-

i) A9 promoter linked to the coding region of the *A. thaliana* A9 gene, such that anti-sense A9 RNA is expressed;

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ii) A3 promoter driving expression of anti-sense A9, either from the A9 cDNA or from the *A. thaliana* A9 gene;

iii) A9 promoter expressing anti-sense RNA to A3, using either the A3 cDNA or the *A. thaliana* A3 gene;

iv) A3 promoter expressing anti-sense RNA to A3, using either the A3 cDNA or the *A. thaliana* A3 gene.

These plasmids could also be transformed into other members of the *Brassicaceae* causing male sterility in the transgenic plants.

Example 4 Construction of chimeric A3-barnase and A9-barnase genes and their expression in transgenic plants.

To demonstrate the utility of the A3 and A9 promoters they are used to drive the expression of the RNase, barnase, in tapetal cells. Use of the barnase gene to create male sterile plants has been described in EP-A-0344029 (Plant Genetic Systems) and has been published by Mariani *et al.*, *Nature* 347, 737-741.

The oligonucleotide primers :-

5′ GGGTCTAGACCATGGCACAGGTTATCAACACGTTTGACGG 3′ and

5′ GTAAAACGACGGCCAGTGCC 3′

are used in a polymerase chain reaction (PCR) to generate a fragment encoding barstar and the mature barnase product from the plasmid pTG2 (Horovitz *et al.*, *J. Mol. Biol.* **216**, 1031-1044 (1990)). The first primer is homologous to

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nucleotides 95-221 bp of Figure 1 in Hartley R.W. *J. Mol. Biol.* **202**, 913-915 (1988). The second primer is homologous to a sequence immediately next to the *Hin*dIII site of pTZ18U (Pharmacia). Barstar is retained on this fragment since active barnase cannot be cloned in the absence of the specific inhibitor barstar (only barnase is expressed in the transgenic plants). The PCR fragment is digested with *Xba*I, *Hin*dIII and cloned into *Xba*I, *Hin*dIII-cut pBluescript forming pWP120 (Fig.13a).

a) Transcriptional fusion of the A9 promoter to barnase

pWP120 is digested with *Xba*I, *Hin*cII and the barnase/barstar fragment cloned into *Xba*I-*Sma*I cut pWP91 forming pWP127 in which the A9 promoter is transcriptionally fused to the mature barnase sequence (Fig. 13b) (pWP91 is identical to pWP80 except that the polylinker region between *Xba*I and *Eco*RI has been replaced with the sites *Spe*I, *Bam*HI, *Sma*I and *Pst*I). This gene fusion is transferred to pBin19 by ligating the *Xho*I fragment of pWP127 to *Sal*I-cut pBin19.

b) Translational fusion of the A9 promoter and gene to barnase

The primers

5′ GGGTCTAGACCATGGTAATTAGATACTATATTGTTTGTAC 3′ and

5′ AATACGACTCACTATAGG 3′

are used in a PCR reaction to generate an A9 promoter fragment from pWP64 (Fig. 6) that contains the entire 5' untranslated region of the A9 gene and has the sequence around the initiating methionine of the A9 gene mutated to an *Nco*I site (the second primer is homologous to a sequence within the pTZ19U vector). This

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fragment is cut with *Hind*III, *Xba*I and cloned into pWP80 or pWP91 replacing the existing A9 promoter fragments in these intermediate vectors. The new intermediate vectors are pWP112 and pWP113 respectively.

pWP120 is cut with *Nco*I, *Hinc*II and the barnase/barstar fragment cloned into *Nco*I, *Sma*I-cut pWP113 forming pWP128 (Fig. 13b). The chimeric gene is then cloned as an *Xho*I fragment into the *Sal*I site of pBin19.

c) Transcriptional fusion of the A3 promoter to barnase

The A9 promoter of pWP127 is replaced with the A3 promoter by cutting pWP127 with *Sal*I, *Xba*I and cloning in the *Sal*I, *Xba*I A3 promoter fragment of pWP88 forming pWP131 (Fig. 13b). The chimeric gene is transferred as a *Kpn*I, *Xho*I fragment into *Kpn*I, *Sal*I-cut pBin19.

The pBin19 derivative plasmids are transformed into *N. tabacum* where expression of barnase in transgenic plants results in the degradation of the tapetal cells of the anther causing complete male sterility. The plants are female fertile. Thus both the A3 and A9 promoters are tapetum-specific and are suitable for driving the expression of any cytotoxic agent within tapetal cells leading to the production of male sterile, but otherwise phenotypically normal transgenic plants. These plasmids could also be transformed into other crop species such as *B. napus*, *Zea mays* and *Hordeum vulgare* leading to male sterility in the transgenic plants.

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Example 5 Construction and expression in transgenic plants of chimeric gene fusions between the A3 and A9 promoters and a β-1,3 glucanase gene.

The temporal pattern of expression of the A3 and A9 genes determined from Northern analysis (Example 1) and promoter-GUS fusions (Example 2) show that both promoters are active at stages of anther development prior to the release of microspores from tetrads. Thus either promoter is suitable for driving the premature expression of β-1,3 glucanase in anthers leading to male sterility, as previously discussed.

cDNAs encoding an *N. tabacum* basic β-1,3 glucanase has been described by Shinshi *et al.* (*PNAS* **85** 5541-5545 (1988)) and Neale *et al.* (*Plant Cell* **2** 673-684 (1990)). This enzyme is located in the vacuole and it has been suggested that a C-terminal sequence may be responsible for its intracellular location (Van den Bulcke *et al.*, *PNAS* **86** 2673-2677 (1989)). An engineered cDNA for this glucanase is cloned by using two oligonucleotides complementary to the sequence of an *N. tabacum* glucanase (Shinshi *et al.*, 1988, Neale *et al.*, 1990) and using the polymerase chain reaction to isolate a glucanase cDNA from *N. tabacum* mRNA. The first oligonucleotide has sequence :-

5′ CGCTCTAGACCATGGCTGCTATCACACTCCTAGG 3′

This primer contains an *Xba*I and an *Nco*I site followed by a sequence identical to a 5' region of glucanase (positions 7-29 in Shinshi *et al.*, 1989). The second oligonucleotide has sequence:-

5′ GGGCCGCGGTCACCCAAAGTTGATATTATATTTGGGC 3′

This primer has a *Sac*II site followed by a trinucleotide that is a stop codon and

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a sequence complementary to the region that encodes the C-terminus of the mature glucanase (positions 1017-993 in Shinshi *et al.*, 1988). The glucanase is therefore cloned with restriction enzyme sites at both ends for ligation into the intermediate vectors pWP80 and pWP88. Also the enzyme is been engineered so that the C-terminal targeting signal is removed. The enzyme will therefore be secreted rather than be directed to the vacuole when expressed in transgenic plants. The glucanase gene is cloned as an *Xba*I, *Sac*II fragment into *Xba*I, *Sac*II-cut pWP80 forming a transcriptional fusion between the A9 promoter and glucanase forming pDW80PR. An A3-glucanase transcriptional fusion is constructed by replacing the *Sal*I, *Xba*I A9 promoter region of pDW80PR with the *Sal*I, *Xba*I A3 promoter fragment of pWP88 forming and pDW88PR (Fig.14a). A translational fusion of the A9 promoter and gene to the glucanase is made by cloning the glucanase as an *Nco*I, *Sac*II into *Nco*I, *Sac*II-cut pWP112 forming pDW112PR (Fig. 14b). The chimeric genes in pDW80PR and pDW112PR are transferred as *Sac*I, *Eco*RV fragments into *Sac*I, *Sma*I-cut pBin19 and the chimeric gene in pDW88PR transferred as a *Sal*I, *Eco*RV fragment into *Sal*I, *Sma*I-cut pBin19. The pBin19 derivatives are transformed into *N. tabacum*. Callose surrounding the microsporocytes prematurely disappears in the transgenic plants causing male sterility. These plasmids could also be transformed into other crop species such as *B. napus*, *Zea mays* and *Hordeum vulgare* leading to male sterility in the transgenic plants.

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CLAIMS

1. Recombinant or isolated DNA comprising a promoter which naturally drives expression of a gene encoding an 11.6 or 12.9 kDa tapetum protein in *Arabidopsis thaliana* or an equivalent protein in another member of the family *Brassicaceae*.

2. DNA as claimed in claim 1 comprising a promoter which drives expression of a 10.3 kDa tapetum protein in *Brassica napus*, which is equivalent to the 11.6 kDa tapetum protein of *A. thaliana*.

3. DNA as claimed in claim 1, including the promoter 5' to the coding region of the sequence shown in Figure 4.

4. DNA as claimed in claim 1, including the promoter 5' to the coding region of the sequence shown in Figure 7.

5. DNA as claimed in any one of claims 1 to 4, wherein the promoter is operatively linked to DNA which, when expressed, causes male sterility in a plant.

6. DNA as claimed in claim 5, wherein the male sterility DNA encodes a lytic enzyme.

7. DNA as claimed in claim 6, wherein the lytic enzyme causes lysis of nucleic acid, protein, carbohydrate or lipid.

8. DNA as claimed in claim 7, wherein the lytic enzyme is a ribonuclease or a deoxyribonuclease.

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9. DNA as claimed in claim 6, wherein the lytic enzyme causes lysis of a carbohydrate.

10. DNA as claimed in claim 9 wherein the lytic enzyme is glucanase.

11. DNA as claimed in claim 10 including a signal sequence in a translational fusion with the glucanase coding sequence.

12. DNA as claimed in claim 6, wherein the lytic enzyme causes lysis of a protein.

13. DNA as claimed in claim 12, wherein the proteolytic enzyme is actinidin or papain.

14. DNA as claimed in claim 5, wherein the male sterility DNA codes for RNA which is antisense to RNA normally found in a plant tapetum cell.

15. DNA as claimed in claim 14, wherein the male sterility DNA codes for RNA which is antisense to RNA encoding the 11.6 or 12.9 kDa tapetum protein in *Arabidopsis thaliana* or an equivalent protein in another member of the family *Brassicaceae*.

16. Antisense nucleic acid which includes a transcribable strand of DNA complementary to at least part of the strand of DNA that is naturally transcribed in a gene encoding an 11.6 or 12.9 kDa tapetum protein in *Arabidopsis thaliana* or an equivalent protein in another member of the family *Brassicaceae*.

17. Antisense nucleic acid as claimed in claim 16 wherein transcription is under the control of a constitutive promoter.

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18. Antisense nucleic acid as claimed in claim 16 wherein transcription is under the control of a tapetum-specific promoter.

19. DNA as claimed in claim 5, wherein the male sterility DNA codes for RNA which is capable of cleavage of RNA normally found in a plant tapetum cell.

20. DNA as claimed in claim 19, wherein the male sterility DNA codes for RNA which is capable of cleavage of RNA encoding the 11.6 or 12.9 kDa tapetum protein in *Arabidopsis thaliana* or an equivalent protein in another member of the family *Brassicaceae*.

21. DNA encoding a ribozyme capable of specific cleavage of RNA encoded by a gene encoding an 11.6 or 12.9 kDa tapetum protein in *Arabidopsis thaliana* or an equivalent protein in another member of the family *Brassicaceae*. Such ribozyme-encoding DNA would generally be useful in conferring male sterility on members of the family *Brassicaceae*.

22. Ribozyme-encoding DNA as claimed in claim 21 wherein transcription is under the control of a constitutive promoter.

23. Ribozyme-encoding DNA as claimed in claim 21 wherein transcription is under the control of a tapetum-specific promoter.

24. DNA capable of specifically disrupting the proper expression of a gene encoding the 11.6 or 12.9 kDa tapetum protein in *Arabidopsis thaliana* or an equivalent protein in another member of the family *Brassicaceae*.

25. DNA as claimed in any one of claims 1 to 24 comprising a 3' transcription regulation sequence.

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26. DNA as claimed in claim 25, wherein the 3' transcription regulation signals are derived from the Cauliflower Mosaic Virus 35S gene.

27. DNA as claimed in any one of claims 1 to 26 which is recombinant and which in the form of a vector.

28. DNA as claimed in claim 27, wherein the vector is a cloning vector and comprises one or more selectable markers.

29. A microbial host cell transfected or transformed with a vector as claimed in claim 27 or 28.

30. DNA as claimed in any one of claims 1 to 28, which includes a marker sequence which enables a plant transformed with the DNA to be distinguished from plants not so transformed.

31. DNA as claimed in claim 30, wherein the marker sequence confers antibiotic or herbicide resistance or codes for glucuronidase.

32. DNA as claimed in claim 31, wherein the marker sequence is under the control of a second promoter, which is not tapetum-specific.

33. DNA as claimed in claim 32, wherein the second promoter is derived from the Cauliflower Mosaic Virus (CaMV) 35S gene.

34. A plant cell including DNA as claimed in any one of claims 1 to 28 and 30 to 33.

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35. A plant or part of a plant at least some of whose cells are as claimed in claim 34.

36. Propagating material from a plant as claimed in claim 35.

BNSDOCID: <WO___9211379A1_I_>

FIG. 1 (1/2)

DNA sequence of the B. napus cDNA clone A3.

```
                S  S  F  C  L  L  L  L  V  V  F  F  L  N  S  Q  P  A  L
TTTCTTCTTTCTGTTTACTCCTCCTCGTCGTCTTCTTCCTCAATTCTCAACCTGCACTCT
      10        20        30        40        50        60

S  L  R  V  P  K  P  Q  S  E  P  A  S  P  Q  T  M  I  D  D
CACTCCGTGTCCCAAAACCGCAGTCAGAACCAGCATCACCACACAAACCATGATCGATGACT
      70        80        90       100       110       120

S  S  P  M  G  M  I  D  H  A  K  S  M  I  A  G  F  F  S  H
CATCTCCAATGGGAATGATCGACCATGCAAAGTCCATGATTGCTGGATTCTTCAGCCACA
     130       140       150       160       170       180
```


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BNSDOCID: <WO 9211379A1_I_>

FIG. 1 (2/2)

```
K  F  P  V  M  G  W  P  F  P  K  Y  P  P  F  T  M  V  N  P
AGTTTCCAGTAATGGGCTGGCCTTTCCCCAAGTACCCACCTTTCACAATGGTCAACCCTA
        190        200        210        220        230        240

N  V  P  T  N  P  S  G  A  Q  E  E  S  E  K  L  P  S  S  P
ACGTTCCAACAAACCCATCTGGAGCTCAAGAGGAATCAGAGAAGCTACCTTCTTCCCCAA
        250        260        270        280        290        300

S  K  L  N  K  A  G  R  N  A  *
GCAAACTTAACAAAGCTGGACGAAACGCATGAAAATTGTTGTTGGAA
        310        320        330        340
```


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FIG. 2a (1/4)

Homology between the DNA sequences of the B.napus E5 and E3 cDNA clones and the A.thaliana A3 gene.

Percent Similarity: 81.7 - At A3 vs Bn E5
80.0 - At A3 vs Bn E3
95.2 - Bn E5 vs Bn E3

```
770 ATGTCGAAAATCTCAAAAGCTTCTTCTCTCTGTTTACTCCTTCTCGTCTT 819   At A3
                  |||  | |||||||  |||||||||||||  ||||||  |
  1 ..............AAAGGTTTCTTCTTTCTGTTTACTCCTCCTCGTCGT 36    Bn E5
                                               ||||||  |||||||
  1 ..................................GCCCTCCTCGTCGTCGT 17    Bn E3

820 CTTCCTCTTCAGTTCCCGACCCGCACTCTCACTCCGTGGCCCAAAACTTC 869
    ||||  ||  |||  |||  |  |||  ||||||||||||||||  ||||||||   |
 37 CTTCTTCCTCAATTCTCAACCTGCACTCTCACTCCGTGTCCCAAAACCGC 86
    ||||||||||  ||||||||||||||||||||||||  ||||||||||||
 18 CTTCTTCCTCAGTTCTCAACCTGCACTCTCACTCCGTCTCCCAAAACCGC 67
```

BNSDOCID: <WO__9211379A1_I_>

FIG. 2a (2/4)

```
870 AATCATCGGAACCAAAATCAGCTCAAACCCTAATGGATGATTCGTCTTCA 919
    | |||   ||||||  |||| | |||||| | || ||||| || ||| ||
 87 AGTCA...GAACCAGCATCACCACAAACCATGATCGATGACTCATCTCCA 133
    |||||   |||| |||||||| ||||||||||||||||||||||||||||
 68 AGTCA...GAACTAGCATCACCTCAAACCATGATCGATGACTCATCTCCA 114

920 ATGAACAAGATCAACTCTGGAAATGCAAAAACCATGATTGCTGGTTTCTT 969
    |||   | |||| ||       |||||||  |||||||||||| |||||
134 ATGGGAATGATCGAC......CATGCAAAGTCCATGATTGCTGGATTCTT 177
    ||||||||||||||       ||||||||  ||||||||||||||||||
115 ATGGGAATGATCGAC......CATGCAAAAGCCATGATTGCTGGATTCTT 158

970 CAGTCACAAGTTTCCATTAAAGGGCTGGCCTTTCCCTAAGTACCCACCTT 1019
    ||| |||||||||||| ||| ||||||||||||||| ||||||||||||
178 CAGCCACAAGTTTCCAGTAATGGGCTGGCCTTTCCCCAAGTACCCACCTT 227
    |||||||||||||||| |||||||||||||||||| |||||||||| |
159 CAGCCACAAGTTTCCACTAATGGGCTGGCCTTTCCCAAAGTACCCACCCT 208
```


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FIG. 2a (3/4)

```
1020 TCCCTATGGTTAACCCTAATATTGCAACAAACCCATCTGGAGCTCAAGAG 1069
     ||  |  ||||| |||||||||  ||  |||||||||||||||||||||||
 228 TCACAATGGTCAACCCTAACGTTCCAACAAACCCATCTGGAGCTCAAGAG 277
     |||||||||||  |||||||||||||||||  |||||||||||||||||||
 209 TCACAATGGTTAACCCTAACGTTCCAACAAAACCATCTGGAGCTCAAGAG 258

1070 GAATCCGCAAAGTTACCTTCTTCTCCAAGCAAAGACAACAAAGATGGACG 1119
     ||||||  |  |||  |||||||||||  ||||||||  |||||||
 278 GAATCAGAGAAGCTACCTTCTTCCCCAAGCAAACTTAACAAAGCTGGACG 327
     ||||||||||||  |||||||||||||||||||||||  |||||||
 259 GAATCAGAGAAGTTACCTTCTTCCCCAAGCAAACTTAACAAAGATGGACG 308
```


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BNSDOCID: <WO__9211379A1_1_>

FIG. 2a (4/4)

```
1120 AAACGCTTGAAGATTAGAGTT.TACTTATTTATATGGTTT....TTACCA 1169
     |||||| |||| |||   |||  |   |||| ||||||||     ||  ||
 328 AAACGCATGAAAATTGTTGTTGGAAACATTTCTATGGTTTTACATTTTCA 377
     |||||||||||||||| ||||||||||||||||||||||||||||||||
 309 AAACGCATGAAAATTGCTGTTGGAAACATTTCTATGGTTTTACATTTTCA 358

1170 TTGCATCAAATAAAAAATGTACCTTTAACAATTAAATGGTAAAAGAAAAA 1219
     ||  |  |||  |||| |||| |||||||||| ||||||||  |
 378 TTAAAATAAAATAAAATTGTATCTTTAACAATTGAATGGTAAGCG..... 422
     ||||||||||   ||||||||||||||||||||||||||||
 359 TTAAAATAAAG..AAATTGTATCTTTAACAATTGAATGGTAA........ 398
```

BNSDOCID: <WO___9211379A1_I_>

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FIG. 2b

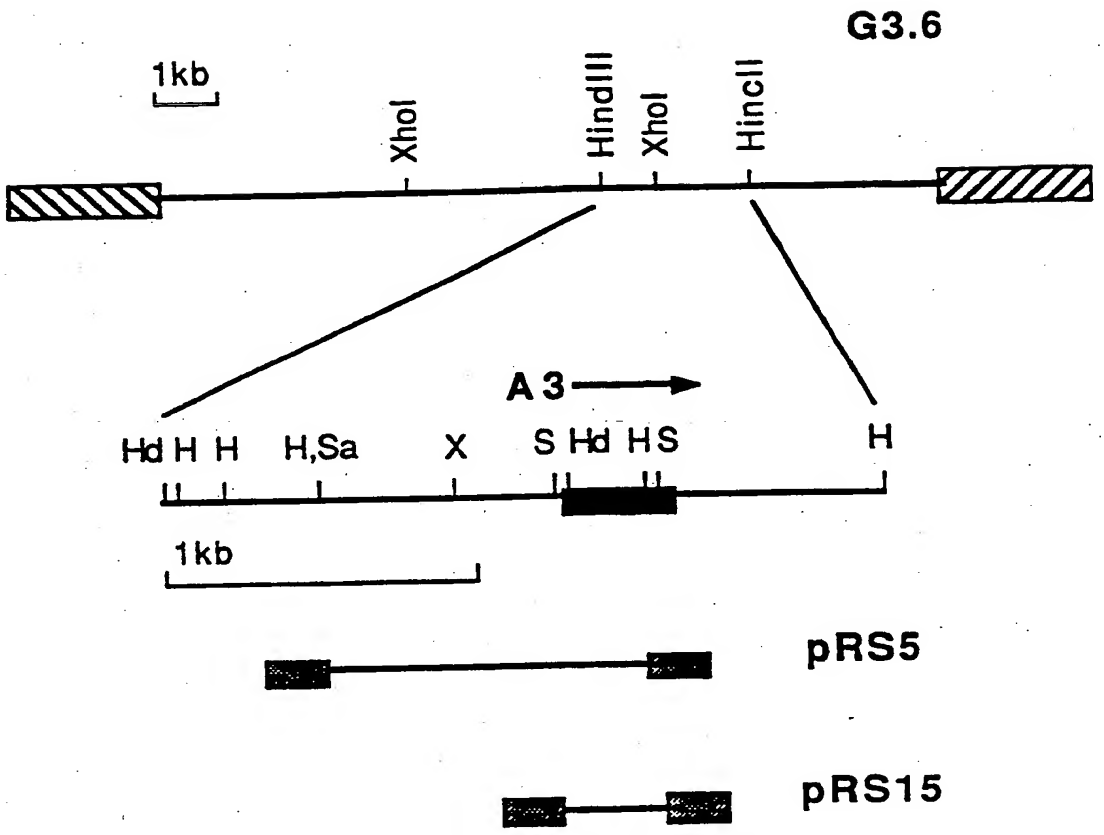
Alignment of the putative polypeptides encoded by B.napus E5 and E3 cDNAs and the A.thaliana A3 gene.

```
* :=>  match across all seqs.
. :=>  conservative substitutions

At A3  MSKISKASSLCLLLLVFFLFSSRPALSLRGPKLQSSEPKSAQTLMDDSSSMNKINSGNAK
Bn E5  -----KVSSFCLLLLVVFFLNSQPALSLRVPKPQS-EPASPQTMIDDSSPMGMID--HAK
Bn E3  -----------ALLVVVFFLSSQPALSLRLPKPQS-ELASPQTMIDDSSPMGMID--HAK
                  **.* *...*.****** ** ** *  *.**..****.*..*.  .***

At A3  TMIAGFFSHKFPLKGWPFPKYPPFPMVNPNIATNPSGAQEESAKLPSSPSKDNKDGRNA
Bn E5  SMIAGFFSHKFPVMGWPFPKYPPFTMVNPNVPTNPSGAQEESEKLPSSPSKLNKAGRNA
Bn E3  AMIAGFFSHKFPLMGWPFPKYPPFTMVNPNVPTKPSGAQEESEKLPSSPSKLNKDGRNA
        .**********..**********.*****..*.********.******** **.****
```

FIG. 3



Key to DNA sequences

Arabidopsis

lambda Dash

pTZ vector

A3 coding region

BNSDOCID: <WO__9211379A1_I_>

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FIG. 4 (1/5)

DNA sequences of the A. thaliana A3 gene.

```
GTCGACACTATTTGTTAATCAGTAGACTCATTTGTGCTGCCTTTGGCTTCTTTTCGTCGG
        10        20        30        40        50        60

AAAAACAGCTGAATGTGACTCTAATTTCGGCGGCAGCAAACGCGGCGGCGTTCATGGCGG
        70        80        90       100       110       120

AGGTAGGAAAGAACGGGAACAAACACGCGAGATGGGACAAAATCTGTGACAGATTCGCCA
       130       140       150       160       170       180

CTTACTGTGATCACGGCGCCGGAGCATTAATCGCCGCATTCGCCGGCGTAATCCTTATGC
       190       200       210       220       230       240
```

BNSDOCID: <WO__9211379A1_I_>

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FIG. 4 (2/5)

```
TCATCATCTCCGCCGCGTCAATCTCTCGTCTCGTCCAACCTAATAAATGCTGCTCCACCA
      250       260       270       280       290       300

CCGCATCTCCCTCGGTCGTCCCCTGAAAACATACACACGTGTCACGTTCCCCTGTTATTT
      310       320       330       340       350       360

TCCTTTTCTTTTCTTACTTCTTTGTTTCTTCTTGAGTGTGTGGTGTGTATCTCGAGTGAA
      370       380       390       400       410       420

TGTGATTGATCAGATTGTGTAAGCATTTGGCTCGTCTCTTCTTTGTAAAACTTTCTTCTT
      430       440       450       460       470       480
```

BNSDOCID: <WO___9211379A1_I_>

FIG. 4 (3/5)

```
ATGGTTTATTCTTAAACCAATTCTCCGACTAGGGTTAGGTTACTAAAATATCCATTACCT
      490       500       510       520       530       540

ATACTCGTTATCAATACCCATACTCGTTATATCAACCATAAATCATCTCTCATAGTCATG
      550       560       570       580       590       600

CTCAAATCATGAACCTTTTTAATTCGAATACTGGTTTAGGTAAGAAGTAGAATCCCAACG
      610       620       630       640       650       660

TAAAAAACACATCTCTTCTACAAACTCAAAAATCACTACTATATAAGATTACCAAGAAAC
      670       680       690       700       710       720
```


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FIG. 4 (4/5)

```
                                                                  M  S  K  I
CCCATAAAACACAATCACAAACAAGAGCTCAAAAACACCAAAGCAAACAATGTCGAAAAT
      730        740        750        760        770        780

  S  K  A  S  S  L  C  L  L  L  L  V  F  F  L  F  S  S  R  P
CTCAAAAGCTTCTTCTCTCTGTTTACTCCTTCTCGTCTTCTTCCTCTTCAGTTCCCGACC
      790        800        810        820        830        840

  A  L  S  L  R  G  P  K  L  Q  S  S  E  P  K  S  A  Q  T  L
CGCACTCTCACTCCGTGGCCCAAAACTTCAATCATCGGAACCAAAATCAGCTCAAACCCT
      850        860        870        880        890        900

  M  D  D  S  S  S  M  N  K  I  N  S  G  N  A  K  T  M  I  A
AATGGATGATTCGTCTTCAATGAACAAGATCAACTCTGGAAATGCAAAAACCATGATTGC
      910        920        930        940        950        960

  G  F  F  S  H  K  F  P  L  K  G  W  P  F  P  K  Y  P  P  F
TGGTTTCTTCAGTCACAAGTTTCCATTAAAGGGCTGGCCTTTCCCTAAGTACCCACCTTT
      970        980        990       1000       1010       1020
```

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BNSDOCID: <WO__9211379A1_I_>

FIG. 4 (5/5)

```
  P   M   V   N   P   N   I   A   T   N   P   S   G   A   Q   E   E   S   A   K
CCCTATGGTTAACCCTAATATTGCAACAAACCCATCTGGAGCTCAAGAGGAATCCGCAAA
      1030        1040        1050        1060        1070        1080

  L   P   S   S   P   S   K   D   N   K   D   G   R   N   A   *
GTTACCTTCTTCTCCAAGCAAAGACAACAAAGATGGACGAAACGCTTGAAGATTAGAGTT
      1090        1100        1110        1120        1130        1140

TACTTATTTATATGGTTTTTACCATTGCATCAAATAAAAAATGTACCTTTAACAATTAAA
      1150        1160        1170        1180        1190        1200

TGGTAAAAGAAAAAAGATATTTATATATCATCAGCTCGAAGCGATTTTGTTATGACAGTT
      1210        1220        1230        1240        1250        1260

ACAGGAATATTAACAA
      1270
```


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FIG. 5 (1/2)

DNA sequence of the B. napus cDNA clone A9.

```
          M  E  F  L  K  S  F  T  T  I  L  F  V  M  F  L  A
TAAACAAAATGGAATTTCTCAAATCCTTTACAACTATTCTCTTTGTAATGTTTCTGGCCA
        10         20         30         40         50         60

M  S  A  L  E  T  V  P  M  V  R  A  Q  Q  C  L  D  N  L  S
TGAGCGCTCTGGAGACCGTACCTATGGTTCGAGCTCAACAATGCCTAGACAATTTGAGCA
        70         80         90        100        110        120

N  M  Q  V  C  A  P  L  V  L  P  G  A  V  N  P  A  P  N  S
ATATGCAGGTGTGTGCGCCGCTGGTTCTGCCTGGTGCAGTCAATCCAGCCCCGAATTCAA
       130        140        150        160        170        180

N  C  C  I  A  L  Q  A  T  N  K  D  C  I  C  N  A  L  R  A
ATTGCTGCATTGCTCTCCAAGCAACTAACAAAGATTGTATATGTAACGCCCTTCGAGCAG
       190        200        210        220        230        240
```

FIG. 5 (2/2)

```
A  T  T  F  T  T  T  C  N  L  P  S  L  D  C  G  I  T  I  *
CCACCACATTTACCACTACTTGCAACCTCCCCTCTTTAGATTGTGGTATAACCATATGAG
      250        260        270        280        290        300

TGGTTTCAGCAACGGTCAGTTCCGAGGATTTGGGGAGTTTGGTCTGCAAAAGACAACAAG
      310        320        330        340        350        360

AATAAAAGTATTAAAATAACGAGAAAGTGTGTGTTTTTTTTTAATTTGGTCTGTTGTT
      370        380        390        400        410        420

CGGTTGGTTCAATACTTAAATATGACCCATCAATTAATATCGTTTTCATATTTATTATGG
      430        440        450        460        470        480

TAATATTTTT
      490
```

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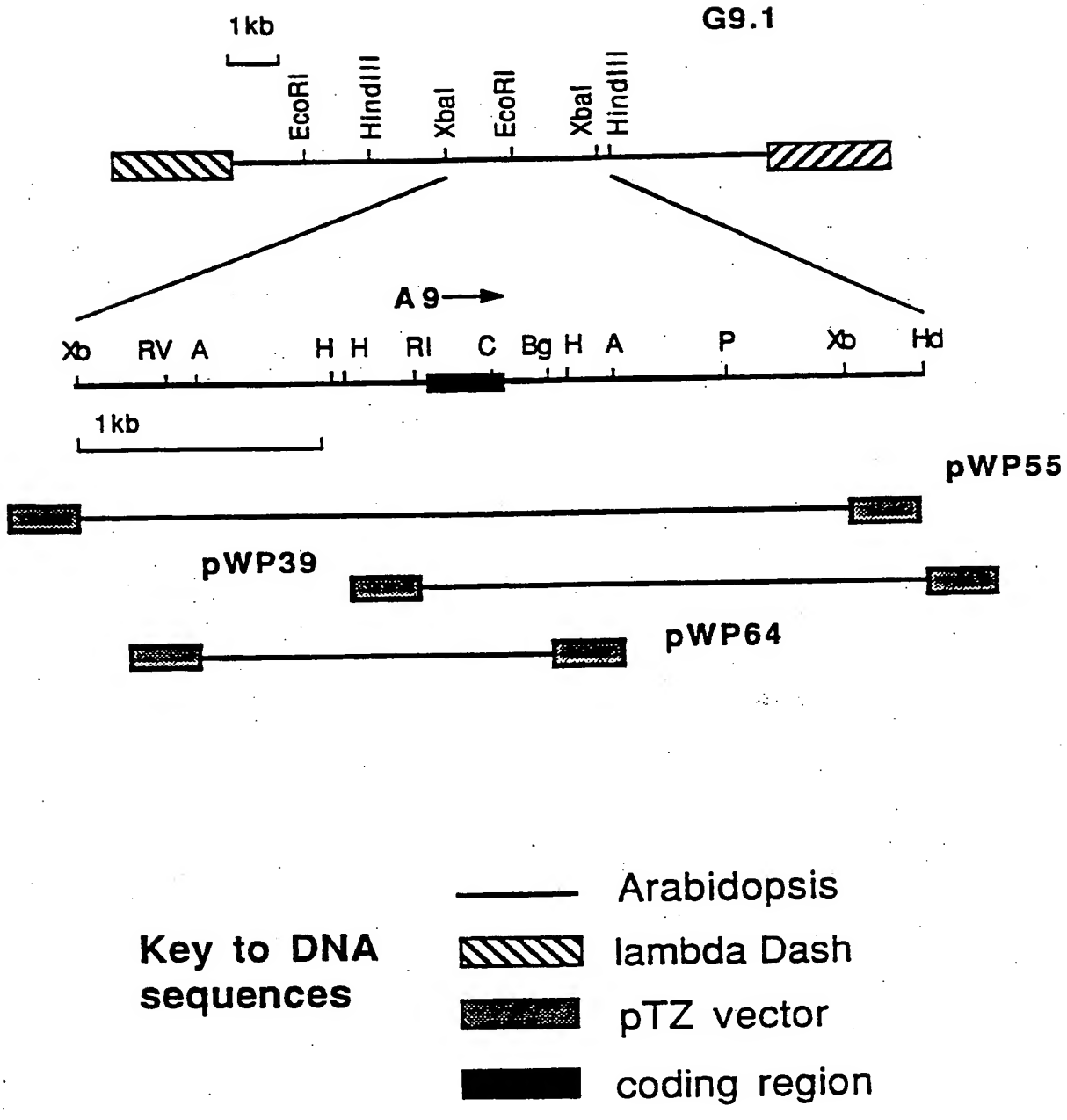
BNSDOCID: <WO___9211379A1_I_>

FIG. 6


1kb
G9.1
EcoRI
HindIII
XbaI
EcoRI
XbaI
HindIII
A 9
Xb RV A H H RI C Bg H A P Xb Hd
1kb
pWP55
pWP39
pWP64
Key to DNA sequences
Arabidopsis
lambda Dash
pTZ vector
coding region

BNSDOCID: <WO__9211379A1_I_>

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FIG. 7 (1/7)

DNA sequences of the A. thaliana A9 gene.

```
TCTAGACATAACGGTGAGAGTTAATATTAAAATTTCAGGCGAGAAAAATGATACTTGAAA
        10        20        30        40        50        60

AATATTATGATCGTTTTGGATATTCCTTACATCGAGTGAATGTTGGTTTGATTCATCTTC
        70        80        90       100       110       120

CAAGTGTTCTGCAAACGTATATTAAAGGTTTATTAACTGGTAAGAGATTAACCGGGTTTT
       130       140       150       160       170       180

GGTTCAGCATATACCATGATTGACTAACTGATCAAATAGTCTTTACTTATTATATAAAGA
       190       200       210       220       230       240

CGATACTATTGGTCATGCTACAAAATCAAGTCATACCATATCCTGAGAATGAATGTGGAG
       250       260       270       280       290       300
```

BNSDOCID: <WO__9211379A1_I_>

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FIG. 7 (2/7)

```
AATCGTTATAAGGCATAAGTGTGGGTATTGATCGTGGTACGAACAACCGCCTTGGCATCA
      310       320       330       340       350       360

ACATTAGCCACGATATCCAACATTTGAAGCATTGCCTATGGCGAGTGTTTGGTTGGTTTT
      370       380       390       400       410       420

GAAACTGATGATGATAACCAGAACGAGAAATGTCTTGTGAAGTATAATGTTCCGATGAAT
      430       440       450       460       470       480

TGGGATTATAATAATGTGTAGACATTGTAGGTTGGTTTTGATGATGATAAGTAATCATTG
      490       500       510       520       530       540


GAGAATTGTCTAACACATGCACTGGAGAATTATTGACTCTACCACGTTCTCTTTGATATT
      550       560       570       580       590       600
```

BNSDOCID: <WO___9211379A1_I_>

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FIG. 7 (3/7)

CTCGATTTTCCTCGTGATTTCATCAGCCTCTCCGAAAAAGTAATTGTATCCACTAGAAC
610 620 630 640 650 660

TTGGGAATCTCCCATCTAATTTATGTATTAGAGAAGTTATAATATTTTGGGGAAATAGA
670 680 690 700 710 720

TTTCTCTACTGATTTTGTTGTGTGACATTATATTTTTATAAGTACATGTTTCTGTTTCG
730 740 750 760 770 780

TATATTGTTGTCGTGGTTGAGTCTTTATTAGAGCATGTAAATATGTTTATGAAATAAGC
790 800 810 820 830 840

AGAAAGGAATTAATTAAACGTATCGAGTGATAAATGCTTTAATGGATTCGAGATTTAGT
850 860 870 880 890 900

BNSDOCID: <WO___9211379A1_I_>

FIG. 7 (4/7)

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ATTCTTAAATTTTTGTTTCATTATCATTGATTATAAAACTAAGTTATGTTGATCTCAAAT
910 920 930 940 950 960

CCTTAATTATGTTCTCCTAAGAAGAGTACAAGTGGTGGGAACGAAAGATGAGTAAAATAC
970 980 990 1000 1010 1020

TAAAAATCTTTTCTCAAAAGTCAAATCGCATTAGTTAACAAAAACAAACCATGTGTTACC
1030 1040 1050 1060 1070 1080

GTCAAATCAATGTGTTTAAAAGATGTTAACCACTAATCAAGCATTTACGTGTAACCGGAT
1090 1100 1110 1120 1130 1140

CAACCGGATTTGGGTTTTGAATATGTTGTGGAGATGTATATAAATGATAAATTAATTGAA
1150 1160 1170 1180 1190 1200

BNSDOCID: <WO__9211378A1_I_>

FIG. 7 (5/7)

```
TATCTTAATTAATCTGTGAAAGAAACTACATCACACACTTTGTTATTTCCCCTAGCTTTT
      1210       1220       1230       1240       1250       1260

AGTTTTTTTATCATGCAAAACTTATGAAGTAACTAGATCAAGATCACAAAAAAAAAAGCAT
      1270       1280       1290       1300       1310       1320

CACTTCACTTCATGACCTAATTATTCTCGAAGCCCAAAACTATTTACATACACTTTTATT
      1330       1340       1350       1360       1370       1380

CTATAAATATAGATGATGGAATTCACCAATCCAAAAGTGAATAAAAAACACAAGTACAAA
      1390       1400       1410       1420       1430       1440

                                M  V  S  L  K  S  L  A  A  I  L  V  A

CAATATAGTATCTAATTAGAATGGTATCTCTAAAGTCCCTTGCTGCTATTCTCGTTGCCA
      1450       1460       1470       1480       1490       1500
```


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BNSDOCID: <WO__9211379A1_I_>

FIG. 7 (6/7)

```
M  F  L  A  T  G  P  T  V  L  A  Q  Q  C  R  D  E  L  S  N
TGTTTCTTGCCACCGGACCTACGGTTCTAGCCCAGCAGTGCAGAGACGAACTGAGCAATG
      1510       1520       1530       1540       1550       1560

V  Q  V  C  A  P  L  L  L  P  G  A  V  N  P  A  A  N  S  N
TGCAGGTGTGCGCGCCGCTGCTTCTGCCCGGTGCGGTCAATCCTGCCGCGAACTCAAATT
      1570       1580       1590       1600       1610       1620

C  C  A  A  L  Q  A  T  N  K  D  C  L  C  N  R  L  R  A  A
GCTGCGCTGCCCTCCAAGCAACTAACAAAGATTGTCTATGTAACCGTCTTCGAGCAGCCA
      1630       1640       1650       1660       1670       1680

T  T  L  T  S  L  C  N  L  P  S  F  D  C  G  K  M  I  H  R
CCACACTTACCTCTCTTTGTAACCTCCCCTCTTTTGATTGTGGTAAGATGATCCATCGAT
      1690       1700       1710       1720       1730       1740

L  K  P  F  L  L  D  F  Y  K  L  F  H  Q  *
TAAAACCTTTTTTACTAGATTTTTATAAATTATTCCATCAATAGTGTTTGTTTTATATTT
      1750       1760       1770       1780       1790       1800
```

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BNSDOCID: <WO__9211379A1_I_>

FIG. 7 (7/7)

```
TTCTCATGATTTTTTGGACTTATGTTTTGTGAACTGTGCAGGCATAAGTGCCTAGTTGA
     1810       1820       1830       1840       1850       1860

CAACATTCAGTTCCGAGGATTTGGGGAGTTTGGTCTGCAAACGACAAGACGAATAAAAT
     1870       1880       1890       1900       1910       1920

AAATAATGAGAAATACACTATTTAGTGTTTT
     1930       1940       1950
```


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BNSDOCID: <WO___9211379A1_1_>

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FIG.8a (1/3)

DNA homology between the B. napus A9 cDNA and the A thaliana A9 gene.

```
                .         .         .         .         .
   1 TAAACAAAATGGAATTTCTCAAATCCTTTACAACTATTCTCTTTGTAATG 50    Bn A9
     |||  | |||||  || ||| || ||| || |  |||||||| |||  |||
1453 TAATTAGAATGGTATCTCTAAAGTCCCTTGCTGCTATTCTCGTTGCCATG 1502  At A9
     .         .         .         .
  51 TTTCTGGCCATGAGCGCTCTGGAGACCGTACCTATGGTTCGAGCTCAACA 100
     |||||  |||                |||| ||||| ||||| ||| || ||
1503 TTTCTTGCC..............ACCGGACCTACGGTTCTAGCCCAGCA 1537
                .         .         .         .         .
 101 ATGCCTAGACAATTTGAGCAATATGCAGGTGTGTGCGCCGCTGGTTCTGC 150
      |||  |||| |  |||||||| |||||||||| |||||||| ||||||
1538 GTGCAGAGACGAACTGAGCAATGTGCAGGTGTGCGCGCCGCTGCTTCTGC 1587
```

FIG. 8a (2/3)

```
 151 CTGGTGCAGTCAATCCAGCCCCGAATTCAAATTGCTGCATTGCTCTCCAA 200
     | |||||  |||||||| |||  ||||  ||||||||||||   |||  ||||||
1588 CCGGTGCGGTCAATCCTGCCGCGAACTCAAATTGCTGCGCTGCCCTCCAA 1637

 201 GCAACTAACAAAGATTGTATATGTAACGCCCTTCGAGCAGCCACCACATT 250
     ||||||||||||||||||  ||||||||   ||||||||||||||||||  |
1638 GCAACTAACAAAGATTGTCTATGTAACCGTCTTCGAGCAGCCACCACACT 1687

 251 TACCACTACTTGCAACCTCCCCTCTTTAGATTGTGGTATAACCAT..... 295
     ||||  ||  |||  ||||||||||||||  ||||||||||   |  ||
1688 TACCTCTCTTTGTAACCTCCCCTCTTTTGATTGTGGTAAGATGATCCATC 1737

 296 .......ATGAGTGGTTTCAGCAACGGTCAGTTCCGAGGATTTGGGGAGT 338
           |    |  |  ||        |  |      ||     | ||
1738 GATTAAAACCTTTTTTACTAGATTTTTATAAATTATTCCATCAATAGTGT 1787
```

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BNSDOCID: <WO__9211379A1_I_>

FIG. 8a (3/3)

```
339 TTGGTCTGCAAAAGACAACAAGAATAAAAGTATTAAAATAACGAGAAAGT 388
    |||  |  |   |    |     ||  ||  |    |  |  |  |   |  |||
788 TTGTTTTATATTTGTTCTCATGATTTTTTGGACTTATGTTTTGTGAACTG 1837

389 GTGTGTGTTTTTTTTTAATTTGGTCTGTTGTTCGGTTGGTTCAATACTTA 438
        |   |   |    | |||  |     |  |  |  |   | |
838 TGCAGGCATAAGTGCCTAGTTGAACAACATTCAGTTCCGAGGATTTGGGG 1887

439 AATATGACCCATCAATTAATATCGTTTTCATATTTATTATGGTAATATTT 488
    | | ||  |    ||  |  |      | | ||  | ||  |  | ||
888 AGTTTGGTCTGCAAACGACAAGACGAATAAAATAAAATAATGAGAAATAC 1937

489 TT 490

938 AC 1939
```


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FIG. 8b

Alignment of the putative polypeptides encoded by B. napus A9 cDNA and the A. thaliana A9 gene

ercent Similarity: 72.8

```
n A9  1 MVSLKSLAAILVAMFLA.....TGPTVLAQQCRDELSNVQVCAPLLLPGA 45
        |  |||   ||  ||||     | | | |||| | ||| |||||| ||||
t A9  1 MEFLKSFTTILFVMFLAMSALETVPMVRAQQCLDNLSNMQVCAPLVLPGA 50

    46 VNPAANSNCCAALQATNKDCLCNRLRAATTLTSLCNLPSFDCGKMIHRLK 95
       |||| ||||| |||||||||| || |||||| |  ||||| |||  |
    51 VNPAPNSNCCIALQATNKDCICNALRAATTFTTTCNLPSLDCGITI.... 97

    96 LFHQ 99
```

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FIG. 9

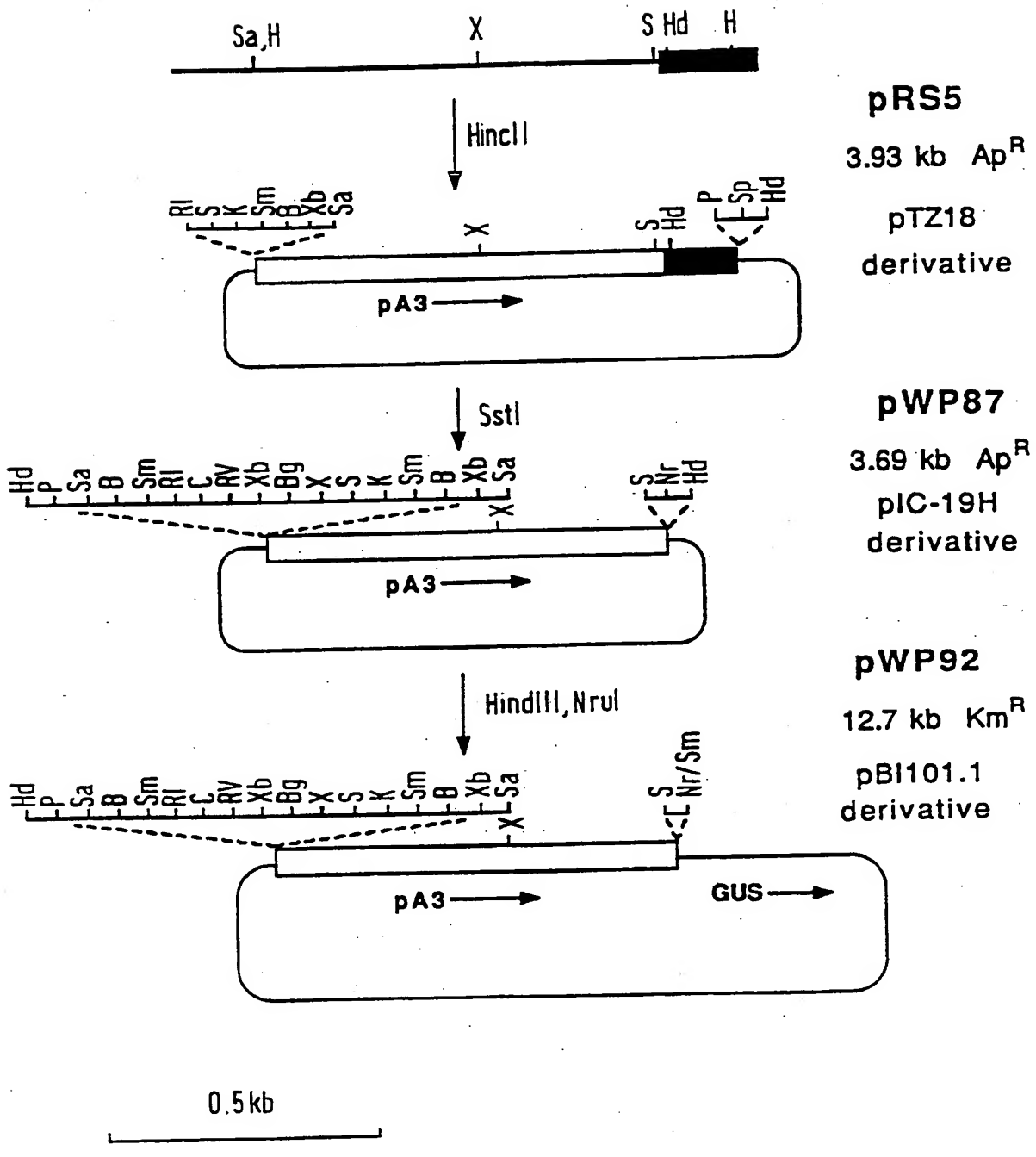

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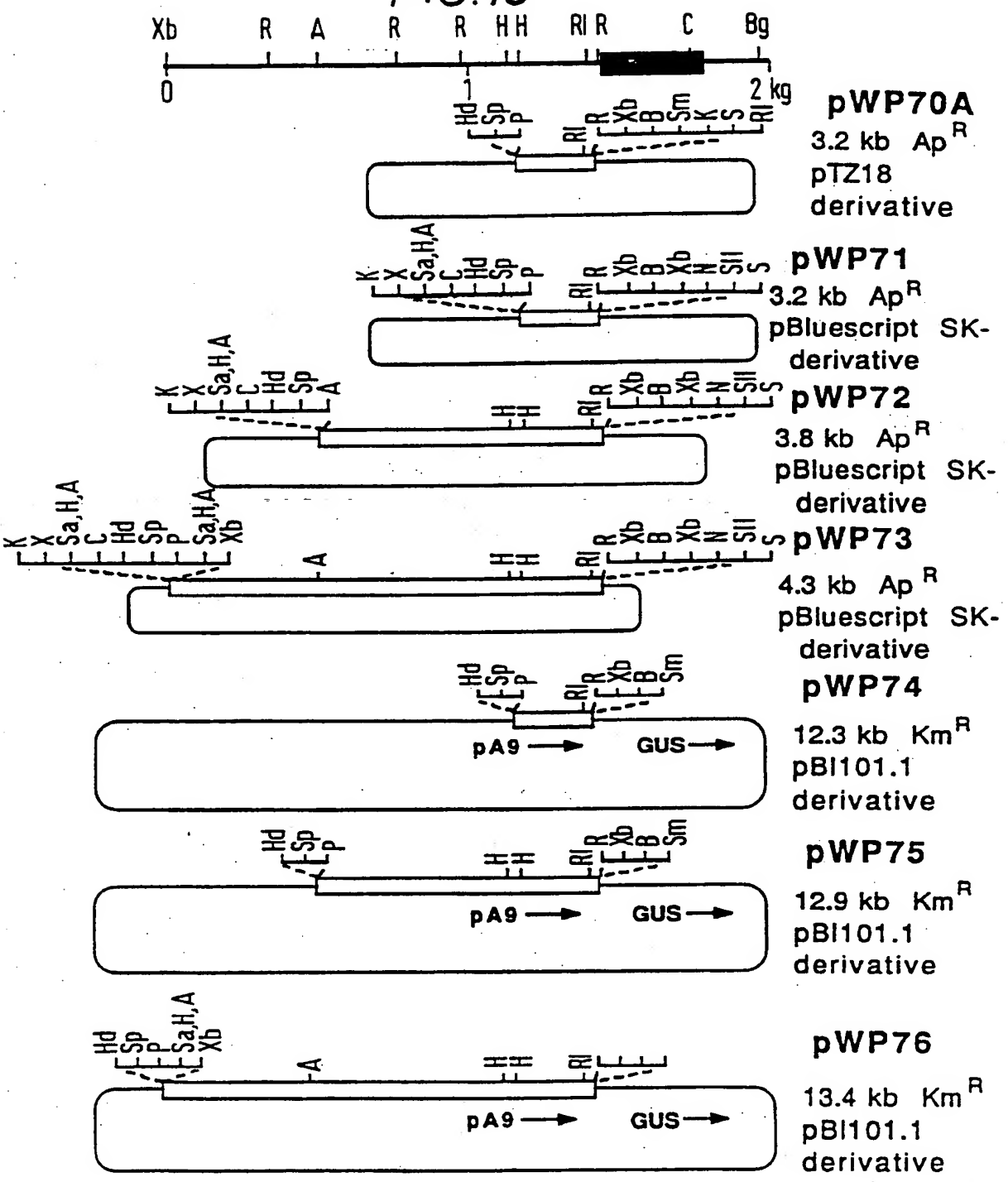
FIG. 10



SUBSTITUTE SHEET

BNSDOCID: <WO___9211379A1_I_>

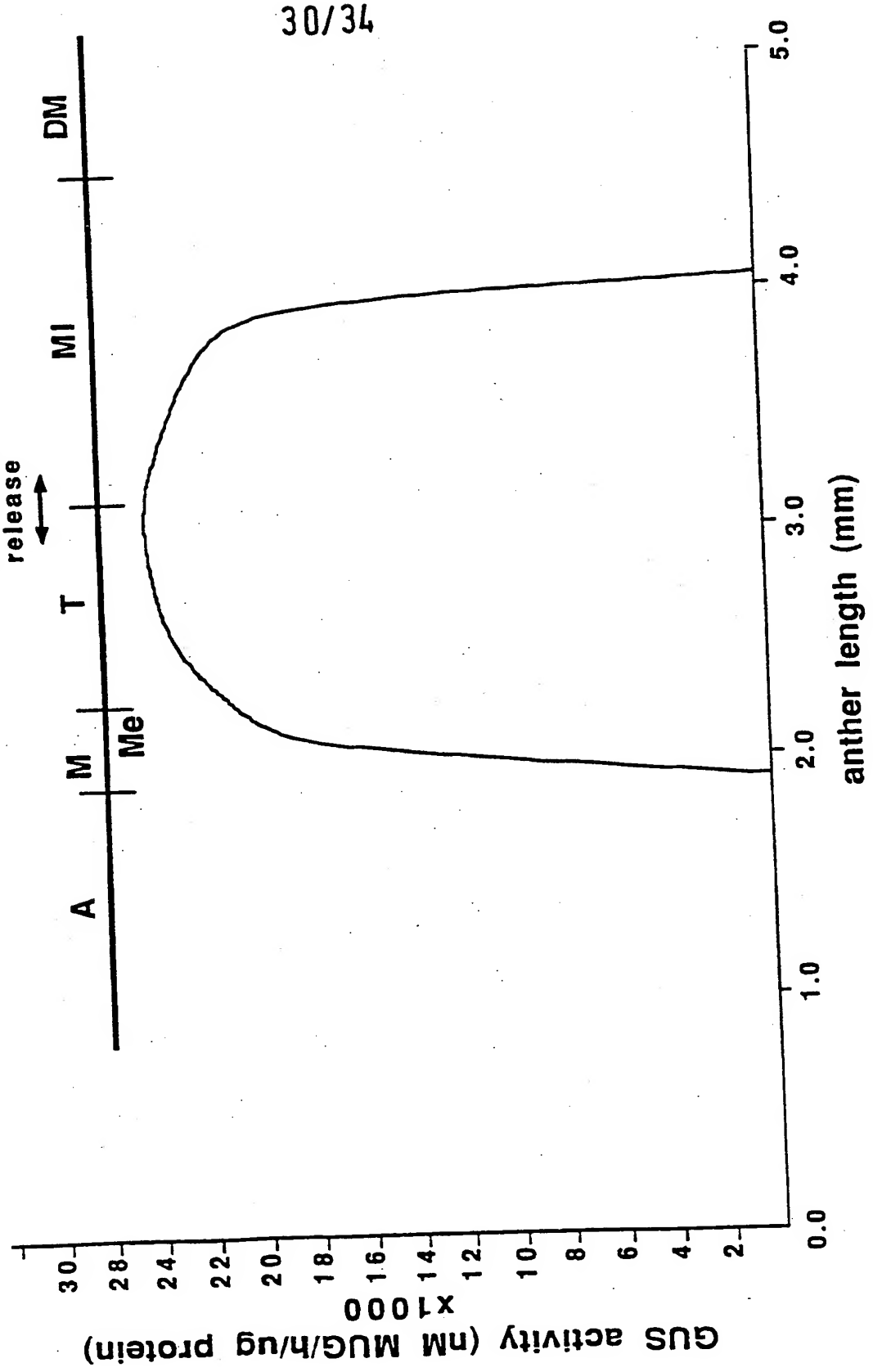
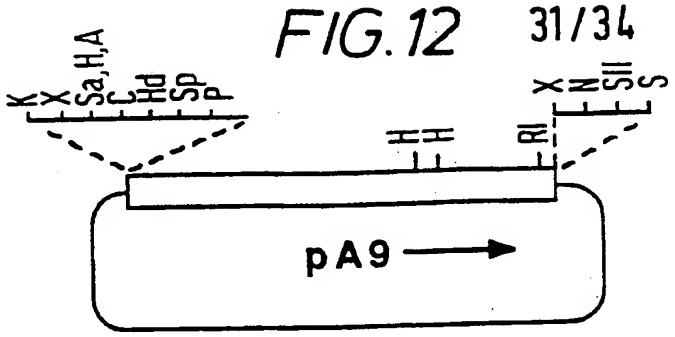
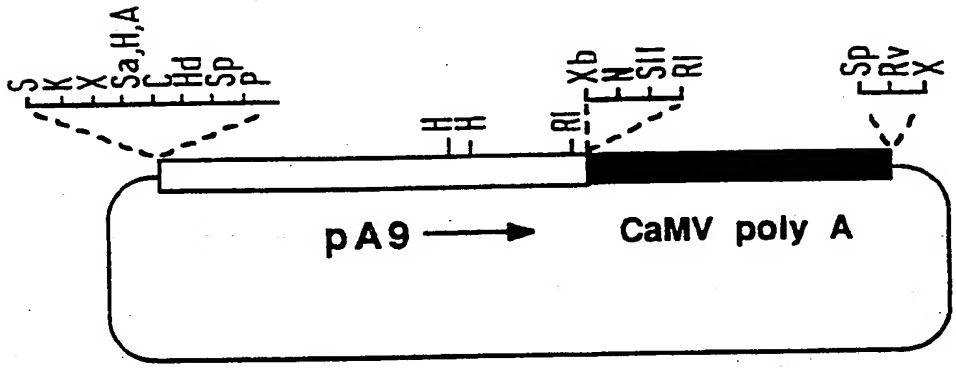


FIG.11 GUS activity in anthers of A9-GUS tobacco plants

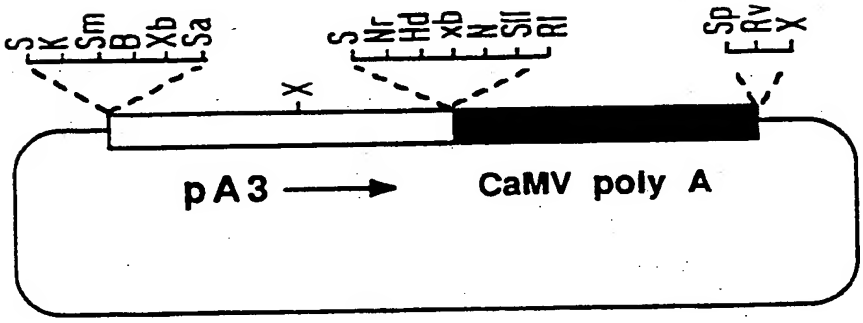
FIG. 12 31/34



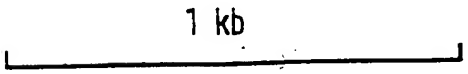
pWP78
3.8 kb Ap^R
pBluescript
derivative



pWP80
3.8 kb Ap^R
pJIT60
derivative



pWP88
3.6 kb Ap^R
pJIT60
derivative



Key to DNA sequences

promoter

polyA signal

FIG. 13a

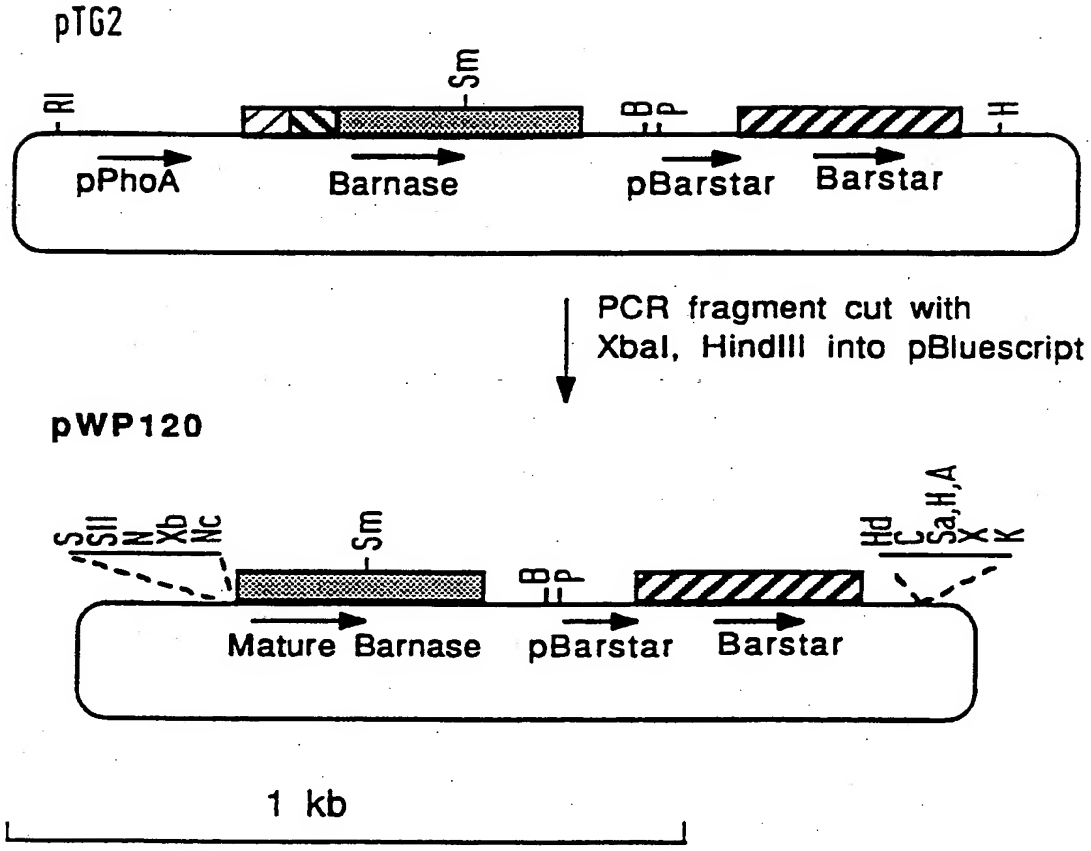

pTG2
RI
Sm
B
P
H
pPhoA
Barnase
pBarstar
Barstar
PCR fragment cut with
XbaI, HindIII into pBluescript
pWP120
S
SII
N
Xb
Nc
Sm
B
P
Hd
C
Sa,H,A
X
K
Mature Barnase
pBarstar
Barstar
1 kb
Signal peptide
N-terminal extension
Mature Barnase
Barstar

FIG.13b 33/34

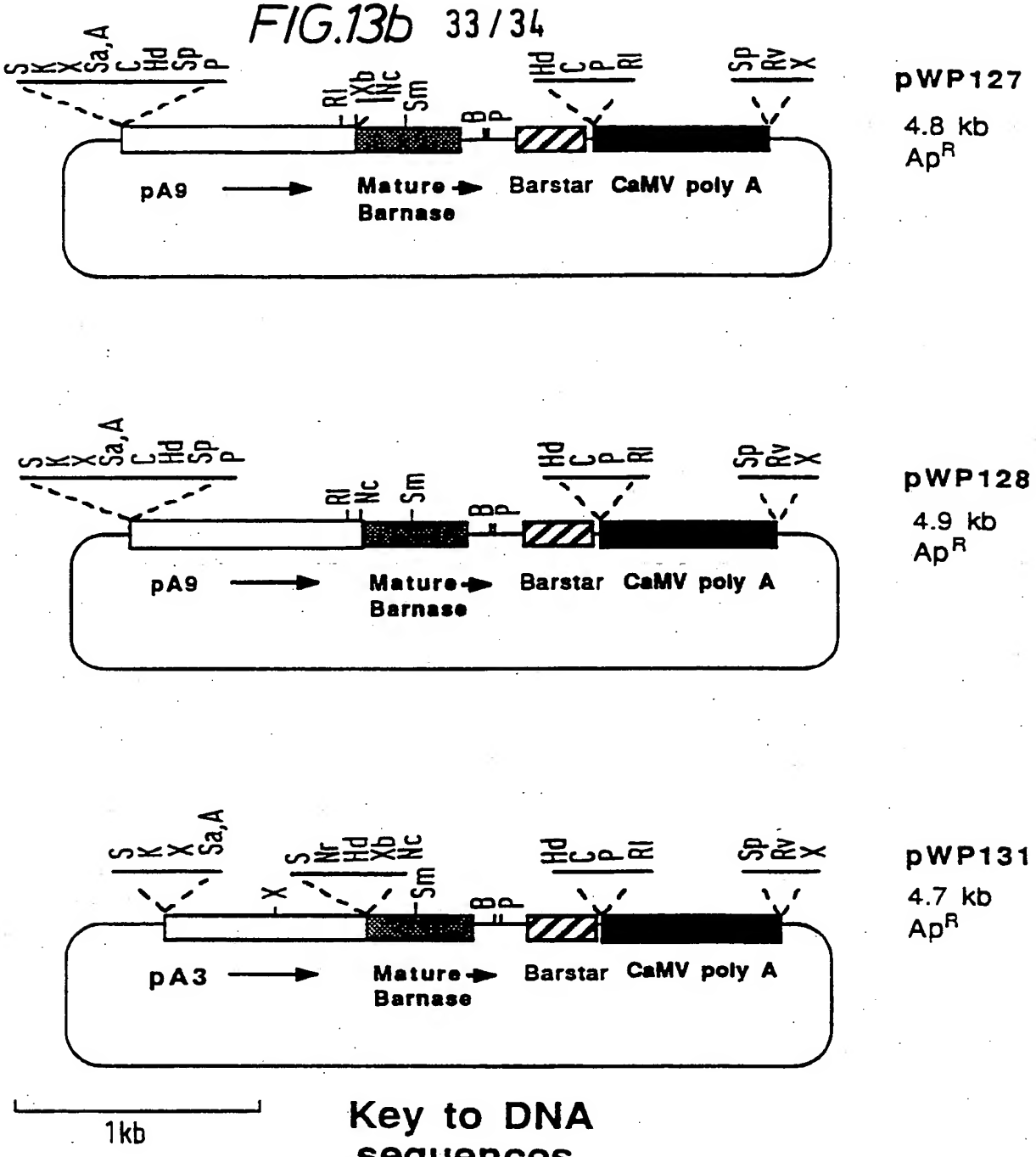

S K X Sa,A C Hd Sp P
RI Xb Nc Sm
B P
Hd C P RI
Sp Rv X
pA9
Mature Barnase
Barstar
CaMV poly A
pWP127
4.8 kb
Ap^R
pWP128
4.9 kb
Ap^R
S K X Sa,A C Hd Sp P
RI Nc Sm
B P
Hd C P RI
Sp Rv X
pA9
Mature Barnase
Barstar
CaMV poly A
pWP131
4.7 kb
Ap^R
S K X Sa,A
S Nr Hd Xb Nc
X
Sm
B P
Hd C P RI
Sp Rv X
pA3
Mature Barnase
Barstar
CaMV poly A
1kb
Key to DNA sequences
Barnase
Barstar
Tapetum-specific promoter
CaMV polyadenylation signal

FIG. 14a

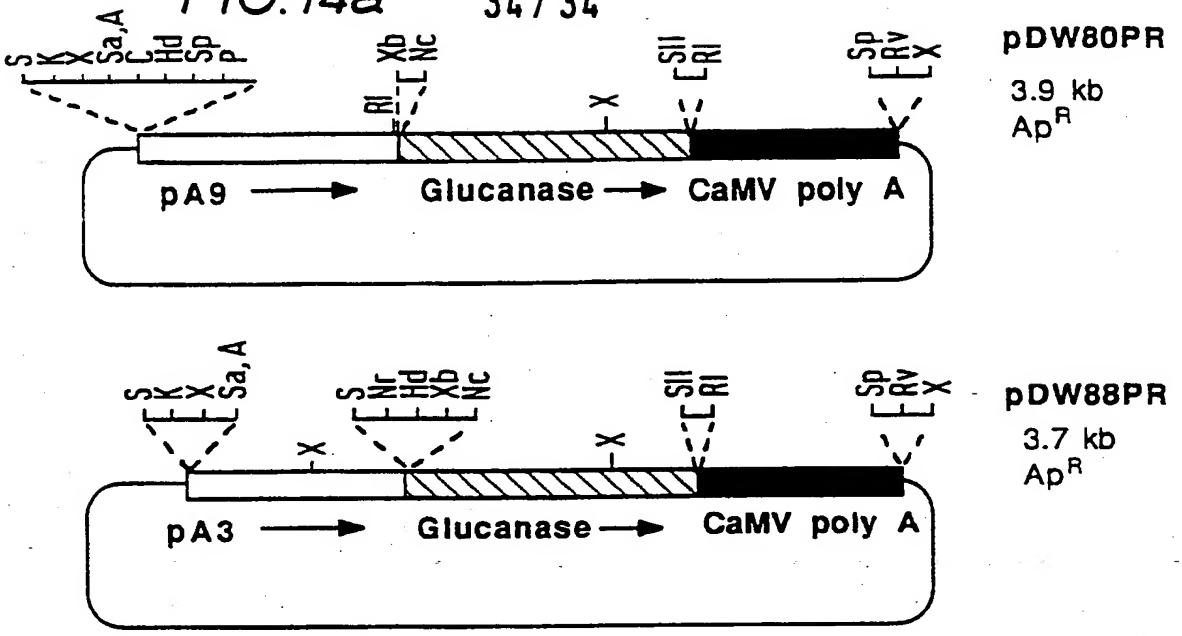
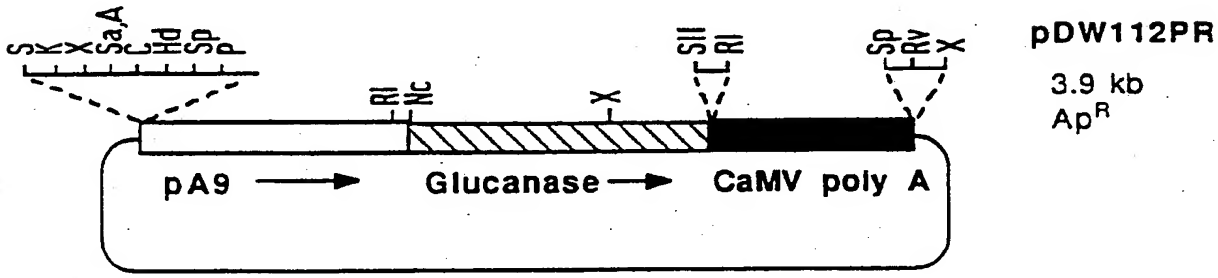
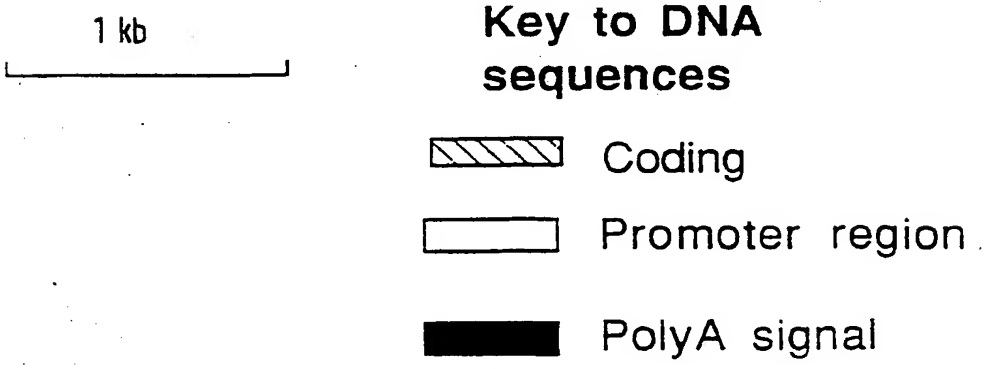


FIG. 14b





INTERNATIONAL SEARCH REPORT

PCT/GB 91/02317

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/29; C12N15/11; C12N9/00
C12N5/10; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	J. EXP. BOT. vol. 41, 1990, SUPPL., P5-3 SCOTT, R., ET AL.: 'Identification of genes exhibiting cell-specific and temporal regulation in developing anthers of Brassica napus' see the abstract P5.09 ---	1-5, 14-18, 25-36
Y	ABSTRACTS VIITH INTERNATIONAL CONGRESS ON PLANT TISSUE AND CELL CULTURE. 1990, JUNE 24-29, AMSTERDAM, NL. page 46; BARGHCHI, M.,ET AL.: 'Genetic engineering of Arabidopsis' see the abstract A2-10 --- -/--	1-5, 14-18, 25-36

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 APRIL 1992	29 APR 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

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Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
O,P, X	J. EXP. BOT. vol. 42, 1991, 238 SUPPL., MEETING APRIL 7-12, 1991 page 46; HODGE, R. P., ET AL.: 'A9 a tapetum-specific gene' see abstract P8.55 ---	1,2,4, 14, 15-18, 25-36
O,P, X	J. EXP. BOTANY vol. 42, 1991, 238 SUPPL.,MEETING APRIL 7-12,1991. page 40; PAUL, W., ET AL.: 'Aspects of the molecular biology of anther development' see abstract P8.24 ---	1-4
O,A	J. EXP. BOTANY vol. 41, 1990, SUPPL., P5-2 ROBERTS, M.,ET AL.: 'Isolation and characterization of pollen specific promoters from Arabidopsis thaliana' see abstract P5-08 ---	1-36
A	WO,A,9 008 828 (PALADIN HYBRIDS) 9 August 1990 see the whole document ---	1-36
A	EP,A,0 344 029 (PLANT GENETIC SYSTEMS) 29 November 1989 see whole document particularly page 4 line 18 - page 7 line 4 ---	1-36
A	NATURE vol. 347, 25 October 1990, pages 737 - 741; MARIANI, C., ET AL.: 'Induction of male sterility in plants by a chimaeric ribonuclease gene' see the whole document ---	1-36

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9102317 SA 55102

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9008828	09-08-90	AU-A- 5037290	24-08-90
		EP-A- 0456706	21-11-91
EP-A-0344029	29-11-89	AU-B- 621113	05-03-92
		AU-A- 3537189	24-11-89
		WO-A- 8910396	02-11-89
		JP-T- 2503988	22-11-90

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